METHIONINE AND SELENIUM TOXICITY[1, 2]

H. L. KLUG, R. D. HARSHFIELD, R. M. PENGRA AND A. L. MOXON
Department of Experiment Station Chemistry,
South Dakota State College, Brookings

FIVE FIGURES

(Received for publication April 14, 1952)

A high-protein ration affords greater protection against selenium poisoning in animals than one of a low-protein content (Moxon, '37; Smith, '39; Smith and Stohlman, '40; Gortner, '40; Lewis, Schultz and Gortner, '40; Rosenfeld and Beath, '46). Of the commercial proteins, crude casein and linseed meal have shown the greatest protection against liver damage in rats. Linseed meal was the only one to give protection to dogs (Moxon, '41) and to poultry (Anderson, Poley and Moxon, '41). The factor in the proteins responsible for this protection has not been identified. Since methionine has been reported (Heppel, Porterfield and Sharpless, '47) as a protective agent against liver damage by various toxic agents, it was suggested that this protein factor might involve methionine. However, the literature reports concerning methionine and liver damage due to selenium toxicity are conflicting.

Smith ('39) concluded that in selenosis the protein-selenium ratio in a diet was more important than the intake level of selenium, and that methionine supplementation did not help.

[1] Approved for publication by the Director of the South Dakota Agricultural Experiment Station as paper 260 of the Journal Series.

[2] This investigation was supported in part by a research grant (RG-611) from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

Copyright 1952
The Wistar Institute of Anatomy and Biology
All rights reserved

Lewis, Schultz and Gortner ('40) reported that a 30% casein diet gave greater survival and less growth retardation against 25 to 50 p.p.m. of selenite selenium than isocaloric diets containing 6% casein. Methionine supplementation afforded similar protection when added to a 6% casein diet, but cystine did not help. A methionine-supplemented 15% arachin diet also gave greater protection against selenium than plain arachin. Smith and Stohlman ('40) concluded that it was the protein (casein) that was protecting against 15 p.p.m. selenite selenium and not the cystine or methionine supplements. Recently, Fels and Cheldelin ('50) reported a partial alleviation of selenium toxicity in yeast and Sellers, You and Lucas ('50), using a methionine-supplemented diet, found a protection against 20 p.p.m. of selenate selenium in the rat only when a 0.05% tocopherol acetate supplement was included in the diet.

This paper presents evidence to show that methionine does not alleviate selenium poisoning in the rat when seleniferous wheat is fed.

EXPERIMENTAL

The control diet used in all these experiments had the following percentage composition:

Wheat, control, 2.44% N	84.25
Casein, commercial, 12.45% N	10.00
Salts IV (Phillips and Hart, '35)	1.00
Yeast, dried Brewer's 8.27% N	1.00
Lard	3.00
A and D feeding oil	0.75

The substitution of seleniferous wheat (assaying 23 p.p.m. selenium and 2% N) for the control wheat in the above diet was the only difference between the control and toxic diets. The methionine supplemented diets consisted of the designated grams of DL-methionine and a sufficient amount of the proper diet to make 100 gm. The animals were kept in individual cages and were fed and watered ad libitum. Table 1 lists animal groups and diets used in the 5 series.

TABLE 1

Animal group data and identification

ANIMALS AND DIETS	SERIES I	SERIES II	SERIES III	SERIES IV	SERIES V
Initial wt. and sex	210–300 gm females	65 gm males	60 gm males	80 gm males	80 gm males
Number per group	2	4	5	10	5
DIETS	GROUPS				
Control	I	I	I	I	I
Control + 2% methionine (M.)	II	II			
Control + 1% M.			II		
Control + 0.5% M.			III		
Control + 0.5% M. + 0.05% tocopherol acetate (TA.)					II
Toxic (19 P.P.M. Se)	III	III	IV	II [1]	III [1]
Toxic + 2% M.	IV	IV			
Toxic + 1% M.			V		
Toxic + 0.5% M.			VI	III [1]	
Toxic + 0.5% M. + 0.05% TA.					IV [1]

[1] The selenium content of this diet was 13 p.p.m. This was made by using 56.2% seleniferous wheat and 28.1% control wheat with the other ingredients of the control diet unchanged.

RESULTS

The growth curves of the rats in the first series are shown in figure 1. Although there were considerable individual variations in response to the diets, the loss in weight caused by the toxic diet was not prevented by the addition of methionine to the diet.

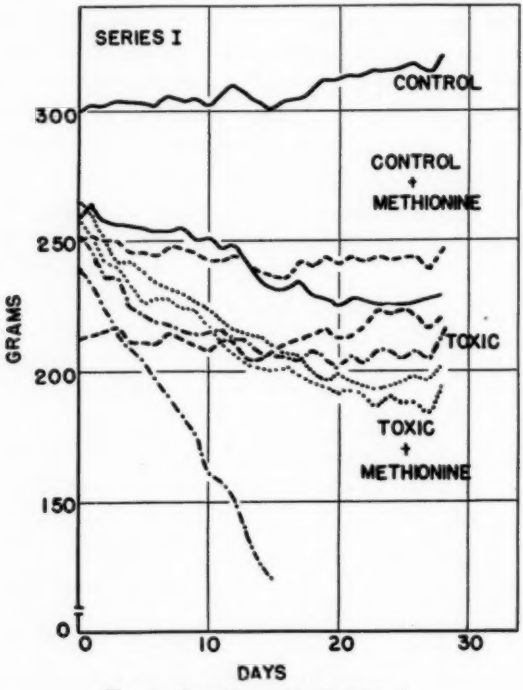

Fig. 1 Growth record of series I.

The animals of series II were young rats that gained steadily in body weight when fed the control diet. As shown in figure 2, the 2% level of methionine inhibited growth. The animals on the seleniferous diet showed the characteristic growth depression with the first death occurring on the 19th day. The group fed the selenium plus methionine diet gained

slightly more, but two of the 4 rats died. The other two were heavier, probably due to some accumulation of ascitic fluid.

The data of figure 3 show that levels of 1.0% and 0.5% methionine do not appreciably affect the growth rate of rats re-

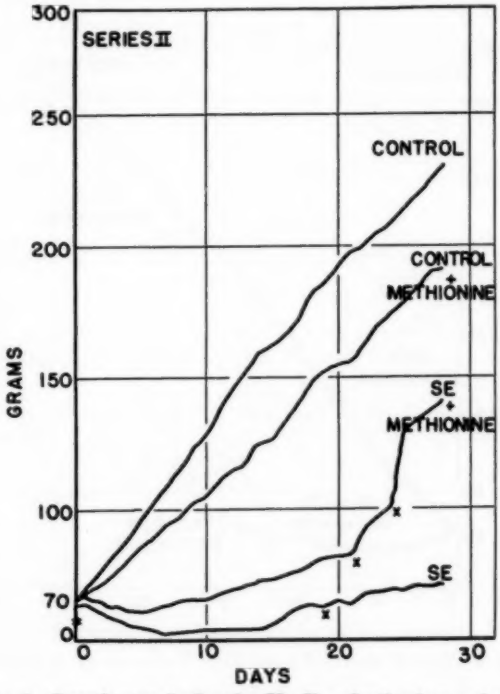

Fig. 2 Growth record of series II. X = death of an animal.

ceiving the control diet. Growth was poor and mortality was high in all groups receiving selenium. In the selenium group without added methionine, only one rat lived to the end of the trial (46 days). All others that received the toxic diets were dead by the 35th day.

Older rats and a lower selenium content in the toxic diets than in the third series were used in series IV. The data

in figure 4 show a slightly better weight gain from the selenium plus methionine diet than from the selenium diet alone, but the early deaths and extensive liver damage previously associated with the former group still occurred. On analysis, the livers from the animals receiving the toxic diet averaged 8.4 p.p.m. of selenium and of the selenium plus methionine

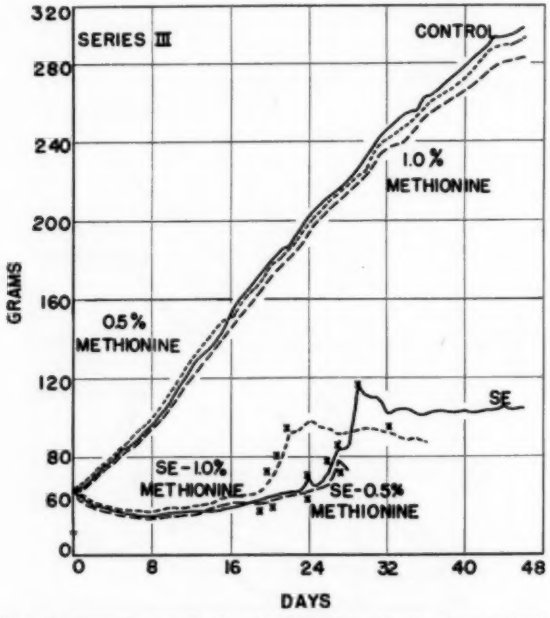

Fig. 3 Growth record of series III. X = death of an animal.

diet, 10.1 p.p.m. Thus, methionine supplementation of the diet had no beneficial effect on reducing the selenium contents of livers.

Further lack of protection by methionine even when supplemented with 0.05% α-tocopherol is shown in figure 5. Four of the 5 animals receiving selenium plus methionine plus tocopherol died during the 5th week of the experiment, whereas three of the selenium fed rats were alive at the termination

of the experiment. A better growth rate was observed in the former group, but autopsies showed severely damaged livers and other characteristic symptoms of selenium poisoning. The results show that supplements of methionine and tocopherol do not prevent the tissue damage caused by naturally occurring selenium.

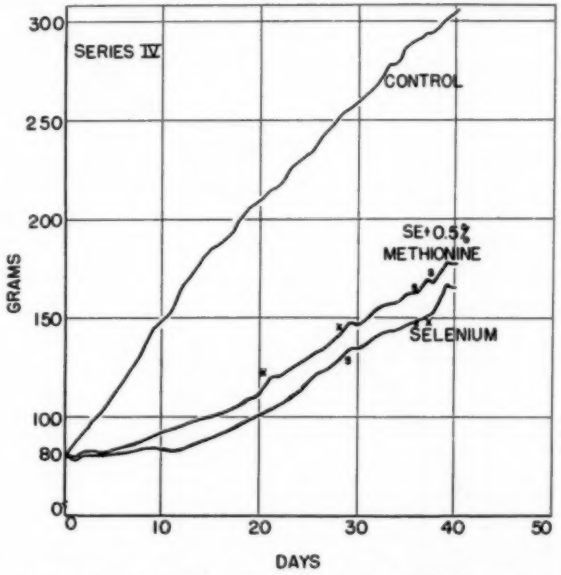

Fig. 4 Growth record of series IV. X = death of animal. S = sacrificed.

The average food consumption for each of the 5 series showed that the presence of methionine in the control diet did not change the food intake nor did it alter the lowered food consumption commonly associated with the ingestion of a seleniferous diet. There was no significant variation in the water intake among the various groups.

The methionine and protein contents of the diet are shown in table 2. The data for methionine have been calculated from Block and Bolling ('45) because selenium interfered in the

analysis of the seleniferous wheat for methionine by the method of McCarthy and Sullivan ('41). The methionine requirement for the rat as given in the literature varies from 0.5% (Treadwell, '48) to 1.2% (Womack and Rose, '41)

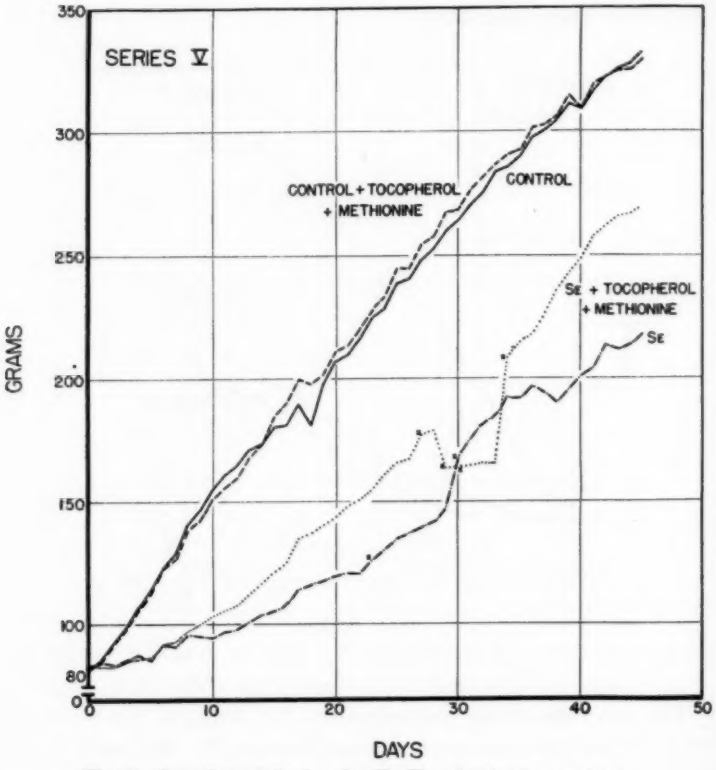

Fig. 5 Growth record of series V. X = death of an animal.

of the diet. The animals of series IV and V that received the selenium diet ate approximately 8 gm of diet or 67 mg of methionine daily. The animals receiving the control diets ate about 15 gm or 114 mg of methionine daily. These diets were not deficient in sulfur-containing amino acids and even

TABLE 2

Methionine and protein contents of diets

COMPONENT	AMOUNT	DIETARY PROTEIN	METHIONINE CONTENT OF MATERIAL		METHIONINE FURNISHED BY MATERIAL PER 100 GM DIET
			Calc.	Found	
	%	%	%	%	gm
Control wheat	84.25	11.1	0.64	0.67	0.539
Casein	10.00	7.9	2.77	2.88	0.277
Yeast	1.00	0.5	1.04	1.00	0.010
Toxic wheat	84.25	9.6	0.52	selenium interferes	0.438
Totals for control diet	95.25 [1]	19.5			0.826
Totals for toxic diets					
Series I, II, III	95.25	18.0			0.725
Series IV, V	95.25	19.5			0.759

[1] Diets of series I, II, III, had 84.25% seleniferous wheat and no control wheat. Diets of series IV and V had 56.2% seleniferous wheat and 28.05% control wheat. See table 1.

if the methionine were not available in the seleniferous rations, 2% supplements of methionine should have sufficiently enriched the diet to mitigate the selenium toxicity if methionine were effective.

Fels and Cheldelin ('48) described the mechanism of selenate toxicity in yeast as one of a competitive inhibition for an enzyme, necessary for the synthesis of methionine from sulfate. Complete reversal of the selenate toxicity was accomplished with sulfate but never with methionine, and the authors acknowledged that "other processes . . . are concerned." Although sulfate sulfur is significant in animal metabolism (Block, Stekol and Loosli, '51), the role of the sulfur-containing amino acids as a sulfur source for the animal body is especially so and any interference in the metabolic scheme becomes important. The failure of the great excess of methionine sulfur compared with the selenium present to prevent the toxicity refutes the idea of a metabolic antagonism or an *in vivo* inactivation of methionine by selenium.

The selenium level used in the first three series was very toxic in relation to the protein content, but reduction of the selenium content of the diet still did not result in any protection from methionine. The danger of an amino acid imbalance is lessened in this series, but it is doubtful if this is critical.

The detoxification of selenium through the respiratory system, possibly as methyl selenide, could involve methionine as a methyl donor, but Lewis, Schultz and Gortner ('40), and Jacobi and Baumann ('42), have failed to show any relationship between methylating agents and the exhaled selenium-containing gases. The selenium compounds present in these gases have not been characterized to date but work is being done on this phase of selenium poisoning in this laboratory.

The data of this experiment show that methionine does not prevent the toxic action of selenium compounds occurring natually in feeds, when fed to rats. Further work is in progress with proteins which show protective action.

SUMMARY

Experiments with varying levels of dietary methionine (0.5% to 2%) have shown that this amino acid is ineffective in the protection of rats against the toxicity of selenium occurring naturally in feeds.

LITERATURE CITED

ANDERSON, H. D., W. E. POLEY AND A. L. MOXON 1941 The effect of dietary protein supplements on the toxicity of seleniferous grains for the chick. Poultry Sci., *20:* 454.

BLOCK, R. J., AND D. BOLLING 1945 The Amino Acid Composition of Proteins and Foods. Charles C. Thomas, Springfield, Ill.

BLOCK, R. J., JAKOB A. STEKOL AND JOHN K. LOOSLI 1951 Synthesis of sulfur amino acids from inorganic sulfate by ruminants. II. Synthesis of cystine and methionine from sodium sulfate by the goat and by the microorganisms of the rumen of the ewe. Arch. Biochem. Biophysics, *33:* 353.

FELS, I. G., AND V. H. CHELDELIN 1948 Methionine in selenium poisoning. J. Biol. Chem., *176:* 819.

———— 1950 Biochemical basis of selenate toxicity in yeast. Ibid., *185:* 803.

GORTNER, R. A., JR. 1940 Chronic selenium poisoning of rats as influenced by dietary protein. J. Nutrition, *19:* 105.

HEPPEL, L. A., V. T. PORTERFIELD AND N. E. SHARPLESS 1947 Toxicology of ethylene dichloride. J. Pharm. Exp. Therap., *91:* 385.

JACOBI, H. P., AND C. A. BAUMANN 1942 The biochemical defect in choline deficient rats. J. Biol. Chem., *142:* 65.

LEWIS, H. B., J. SCHULTZ AND R. A. GORTNER, JR. 1940 Dietary protein and the toxicity of sodium selenite in the white rat. J. Pharm. Exp. Therap., *68:* 292.

MCCARTHY, T. E., AND M. X. SULLIVAN 1941 A new and highly specific colorimetric test for methionine. J. Biol. Chem., *141:* 871.

MOXON, A. L. 1937 Alkali disease. S. Dak. Agri. Exp. Sta. Bull. No. 311, p. 49.

———— 1941 Ph.D. Thesis, University of Wisconsin, Madison.

PHILLIPS, P. H., AND E. B. HART 1935 The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. J. Biol. Chem., *109:* 657.

ROSENFELD, I., AND O. A. BEATH 1946 The influence of protein diets on selenium poisoning. Am. J. Vet. Research, *7:* 52.

SELLERS, E. A., R. W. YOU AND C. C. LUCAS 1950 Lipotropic agents in liver damage produced by selenium or carbon tetrachloride. Proc. Soc. Exp. Biol. Med., *75:* 118.

SMITH, M. I. 1939 The influence of diet on the chronic toxicity of selenium. U. S. Pub. Health Repts., *54:* 1441.

SMITH, M. I., AND E. F. STOHLMAN 1940 Further observations on the influence of dietary protein on the toxicity of selenium. J. Pharm. Exp. Therap., *70:* 270.

TREADWELL, C. R. 1948 Growth and lipotropism. J. Biol. Chem., *176:* 1149.

WOMACK, W., AND W. C. ROSE 1941 The partial replacement of dietary methionine by cystine for purposes of growth. Ibid., *141:* 375.

SYNTHESIS OF CERTAIN B-VITAMINS IN THE COBALT DEFICIENT SHEEP, WITH SPECIAL REFERENCE TO VITAMIN B_{12}[1]

W. G. HOEKSTRA, A. L. POPE AND P. H. PHILLIPS
Departments of Biochemistry and Animal Husbandry, College of Agriculture, University of Wisconsin, Madison

(Received for publication July 21, 1952)

Abelson and Darby ('49) indicated that radio-cobalt fed to sheep is incorporated into fecal vitamin B_{12}. Using a chick assay for vitamin B_{12} in rumen ingesta, Hale and co-workers ('50b) demonstrated that synthesis of this factor was greatly limited in cobalt-deficient sheep as compared to cobalt-fed sheep. Recent work has shown that vitamin B_{12} is an important intermediary in cobalt metabolism in ruminants (Smith et al., '51; Koch and Smith, '51; Marston, '52; Hoekstra et al., '52). The evidence indicates that cobalt deficiency manifests itself as a vitamin B_{12} deficiency, and that the cobalt is necessary for incorporation by certain rumen organisms into vitamin B_{12} active substances which are required by the host animal. It seemed desirable to investigate further the status of the cobalt-deficient sheep as to rumen synthesis, blood level, and liver storage of vitamin B_{12}.

The work of Ray et al. ('47) showed lower blood levels of certain B-vitamins (B_6, niacin, and riboflavin) in the cobalt-deficient sheep. Hale et al. ('50a) reported that a complete B-vitamin supplement exclusive of vitamin B_{12} reversed the symptoms of a cobalt-deficiency; however, the work of Keener

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

et al. ('50) showed no beneficial value of these B-complex vitamins for the cobalt-deficient sheep. Gall and co-workers ('49) have reported that an altered flora occurs in the cobalt-deficient sheep. There existed the possibility of a general inhibition of rumen synthesis in a cobalt deficiency and this was also investigated in relation to the nicotinic acid and riboflavin content of the rumen ingesta of cobalt-deficient sheep.

EXPERIMENTAL

In December of 1950 a group of spring lambs were fed a basal cobalt deficient ration. The basal ration had the following composition: chopped, mixed, grass and alfalfa hay 49.25%; ground oats 49.25%; urea "262" feeding compound 1.50%. The cobalt contents, in parts per million, of these three ingredients, were, respectively, 0.06, 0.03 and 0.07. This ration was fed together with a free choice of iodized salt and tricalcium phosphate. Nine lambs were given supplemental cobalt by mixing one ounce of $CoSO_4 \cdot 7H_2O$ in each 100 lb. of salt. Three of these 9 lambs were limited in feed to the amount eaten by three animals on the basal ration only. Some of the remaining lambs were initially fed a supplement of 2% dried fish solubles (very low in vitamin B_{12}) or the ash from an equivalent amount of fish solubles. After 6 months it was found that this level of fish solubles, or its ash, did not prevent nor significantly delay the appearance of cobalt deficiency symptoms. These animals were definitely cobalt-deficient and were transferred to the basal ration. Thus, essentially this experiment consisted of three lots of animals: lot 1, which received ad libitum the basal cobalt-deficient ration; lot 2, which received ad libitum the basal cobalt-deficient ration plus supplemental cobalt; and lot 3, another control lot on the basal plus cobalt ration but limited in amount ingested to approximately that of the lot 1 animals.

Blood samples were taken by jugular vein puncture into citrated tubes. They were immediately refrigerated until assays were made. Assays were set up on the same day the blood samples were collected. Aliquots were diluted with

distilled water according to the expected blood vitamin B_{12} content, and 5 duplicate levels were added to the assay tubes for each sample. This method gave good results for sheep blood.

The livers were obtained at the time of slaughter or death of the animals. In no case was an animal dead for more than two or three hours before the liver was taken. Livers were stored in a frozen state until assays were made. A representative weighed sample of liver was homogenized with water in a Waring Blendor. The homogenized samples were then heated in boiling water for 10–12 minutes, filtered, the protein precipitate washed several times with boiling water, and the filtrate made up to a given volume. Aliquots of this filtrate were diluted according to the concentration of vitamin B_{12} in the sample. This non-enzymatic method of releasing vitamin B_{12} from livers gives good liberation according to Scheid et al. ('51a, b) and unpublished data at Wisconsin. To determine the alkali-stable vitamin B_{12} activity of the livers an aliquot of the original filtrate (representing a 1:200 dilution) was taken to approximately pH 12.5 with 1 N KOH, autoclaved for 30 minutes at 15 lb. pressure, adjusted to the pH of the media (6.5), diluted to give a final dilution of 1:400, and then assayed along with the original liver samples.

Rumen samples were also collected at slaughter. Representative samples of the contents were homogenized with water in a Waring Blendor and stored in a frozen state until assayed. The vitamin B_{12} of weighed aliquots of these samples was released by incubation with takadiastase and papain at pH 4.5 in acetate buffer. The samples were steamed briefly, adjusted to pH 6.5, filtered, and then diluted to a suitable volume for vitamin B_{12} assay. Nicotinic acid and riboflavin were also determined on the homogenized samples. Samples of the homogenized rumen ingesta were dried at 100°C. and the vitamin concentrations reported on dry weight.

Vitamin B_{12} was determined microbiologically using *Lactobacillus leichmannii* 4797 by the method of Thompson et al. ('50). Five-minute sterilization periods at 15 lb. pressure

were used and the response was measured titrimetrically with 0.1N NaOH after a 72-hour incubation period. Five duplicate levels were set up for each sample. The data usually represented averages of two or more separate assays on each sample. Riboflavin was determined with *Lactobacillus casei* using the modified Snell-Strong medium and procedure as described by Snell ('50) following the recommended acid extraction procedure. The collaborative U.S.P.-A.O.A.C. 1945 media and procedure using *Lactobacillus arabinosus* described by Snell ('50) were used to determine nicotinic acid.

RESULTS

The body weight data are presented in tables 1, 2 and 3 to indicate the severity of the cobalt deficiency. There can be no doubt that the animals in lot 1 were suffering from a cobalt deficiency. Table 1 summarizes the blood vitamin B_{12} concentrations of the three lots of experimental sheep. These data are for a relatively early, but a definite, stage of cobalt deficiency. In later stages of cobalt deficiency, vitamin concentrations in the blood could not be reliably evaluated, since use of too high a concentration of citrated blood inhibited growth of the assay organism. The blood vitamin B_{12} levels of the cobalt-deficient sheep are only about one-fifth of those encountered in animals on an identical ration supplemented with inorganic cobalt. Considerable variation is noted in the blood vitamin B_{12} concentrations of cobalt-fed animals, especially in those given limited amounts of feed. The higher average vitamin B_{12} content of the blood of animals in lot 3 is probably a result of the consumption of larger quantities of salt containing the supplemental cobalt. These animals were being starved, and as a result of intense appetite consumed approximately twice as much salt per head as the animals in lot 2.

Table 2 presents the vitamin B_{12} data of the livers before and after alkali treatment for the three experimental lots. The difference in the two values gives an approximation of the "true" vitamin B_{12} activity. Subtraction of the alkali

stable vitamin B_{12} activity from the total vitamin B_{12} activity for lots 1 and 2 shows that the livers from animals fed cobalt contained, on the average, about 30 times as much "true" vitamin B_{12} activity per gram of moist liver as those from cobalt-deficient animals. In the cobalt-deficient lot nearly

TABLE 1

Comparison of blood vitamin B_{12} values of sheep in early stages of cobalt deficiency with those of cobalt-fed, full-fed sheep and cobalt-fed, limited-fed sheep

LOT	NO. SHEEP	BODY WT. STATUS	VITAMIN B_{12} ACTIVITY OF WHOLE BLOOD ($M\mu G/ML$)[1]
1 (— Co)	16	Lost ave. of 6.8 lb. of max. body wt.	0.47 ± 0.11
2 (plus Co, full-fed)	6	Gained ave. of 27 lb. for period of 10 wks. prior to bleeding	2.3 ± 0.6
3 (plus Co, limited-fed)	3	Lost ave. of 10 lb. of max. body wt.	4.3 ± 1.5

[1] Mean ± standard deviation.

TABLE 2

Comparison of the vitamin B_{12} content of livers of cobalt-deficient sheep with that of cobalt-fed, full-fed sheep and cobalt-fed, limited-fed sheep

LOT	NO. SHEEP	BODY WT. STATUS	AVE. FINAL BODY WT.	TOTAL VITAMIN B_{12} ACTIVITY (μG/GM MOIST LIVER)	ALKALI STABLE VITAMIN B_{12} ACTIVITY (μG/GM MOIST LIVER)
			lb.		
1 (— Co)	9	Lost ave. of 45 lb. prior to death or slaughter	65	0.055 ± 0.015	0.025 ± 0.015
2 (plus Co, full-fed)	6	Ave. daily gain of 0.24 lb. through-out exp. period	133	0.93 ± 0.26	0.009 ± 0.006
3 (plus Co, limited-fed)	3	Lost ave. of 18 lb. prior to slaughter	79	1.24 ± 0.20	0.035 ± 0.002

one-half of the vitamin B_{12} activity was stable to alkali treatment, while only about 1% of the total activity remained in the case of the cobalt-fed, full-fed animals. Lot 1 and especially lot 3 showed a greater amount of alkali stable vitamin B_{12} activity in liver tissue than lot 2.

Results of rumen ingesta analyses presented in table 3 show a much lower synthesis of vitamin B_{12} by cobalt-deficient sheep. A high degree of variation was noted in the cobalt-

TABLE 3

Comparison of the vitamin B_{12}, riboflavin and nicotinic acid content of rumen ingesta of cobalt-deficient sheep with that of cobalt-fed, full-fed sheep and cobalt-fed, limited-fed sheep

LOT	NO. SHEEP	BODY WT. STATUS	AVE. FINAL BODY WT.	VITAMIN B_{12} ACTIVITY (μG/GM DRY WT.)	NICOTINIC ACID (μG/GM DRY WT.)	RIBOFLAVIN (μG/GM DRY WT.)
			lb.			
1 (— Co)	4	Lost ave. of 34 lb. prior to slaughter	60	0.09 ± 0.06	51 ± 16	10.5 ± 1.1
2 (plus Co, full-fed)	5	Ave. daily gain of 0.26 lb. throughout exp.	143	1.3 ± 0.4	59 ± 5	12.6 ± 0.8
3 (plus Co, limited-fed)	3	Lost ave. of 18 lb. prior to slaughter	79	1.3 ± 0.9	59 ± 4	14.3 ± 2.2

fed lots. This was probably because of the variations in intake of cobalt prior to slaughter. Nicotinic acid values do not differ among the three groups. Although there appears to be a slight inhibition of riboflavin synthesis in the cobalt-deficient lot, the values do not differ enough to represent a serious limitation of riboflavin to the sheep.

The data indicate a much lower rumen synthesis of vitamin B_{12}, also a much lower blood level of vitamin B_{12}, and a severe depletion of liver storage of vitamin B_{12} in cobalt-deficient sheep as compared to cobalt-fed sheep.

DISCUSSION

Certain reservations must be considered in accepting vitamin B_{12} values as determined by *L. leichmannii*. It is known that various forms of vitamin B_{12} have different activities for this organism (Broquist et al., '51). A conversion of vitamin B_{12} to a more active form on autoclaving has been reported by Broquist et al. ('51); these authors have discussed the significance of this fact in the interpretation of microbiological assay of natural materials when vitamin B_{12} is used as the standard. The demonstration of the presence of vitamin B_{12f} in rumen ingesta of the sheep by Lewis et al. ('52a, b), and the production of pseudo-vitamins B_{12} by an isolated rumen anaerobe reported by Pfiffner and co-workers ('51) may tend to overevaluate the vitamin B_{12} potency of samples from the cobalt-fed animals. It is known that although both of these materials are active for *L. leichmannii*, vitamin B_{12f} is inactive for the rat, and the pseudo-vitamins B_{12} are inactive for the chick, rat, and man.

Despite these limitations in the determination of vitamin B_{12}, the differences found in this study are so profound that there is little doubt that the cobalt-deficient sheep were severely limited in vitamin B_{12} as compared to the sheep given supplemental cobalt.

The blood vitamin B_{12} concentration obtained for the cobalt-deficient sheep was at the lower range of the values reported by Couch and co-workers ('50; 0.6–0.9 mμg/ml), while in the cobalt-fed sheep the vitamin B_{12} blood level was considerably above their range of values. Our data indicate that the concentration of vitamin B_{12} in blood is dependent on the intake of cobalt, and that literature values for blood of ruminants are subject to this consideration. It should be pointed out that all of the animals in lot 1 were in early stages of cobalt deficiency; the animals' body weight status ranged from a stationary body weight for 6 weeks prior to bleeding to a maximum loss of 23 lb. over a period of 12 weeks prior to bleeding. Meanwhile the cobalt-fed animals were making good body weight gains.

The vitamin B_{12} concentration of the livers of cobalt-deficient sheep compare with values reported for rat liver (Scheid et al., '51a; Lewis et al., '49), while the cobalt-fed sheep liver values compare more with reported beef liver analyses (Schweigert et al., '51). The vitamin B_{12} activity of liver after alkali treatment is generally assumed to be due to the desoxyribosides (Hoffman et al., '49). Scheid, Andrews and Schweigert ('51) have shown that a large portion of the vitamin B_{12} activity of rat liver for *L. leichmannii* is alkali stable. This was also true for the livers of cobalt-deficient sheep in this study. In approximating the "true" vitamin B_{12} activity by deducting the alkali stable activity from the total activity, it is assumed that the two values are strictly additive. This assumption may or may not be valid. Scheid and Schweigert ('51) have demonstrated a higher alkali stable vitamin B_{12} activity of liver from rats supplemented with vitamin B_{12} than from those deficient in vitamin B_{12}. It is not clear why the alkali stable activity was higher in our experiment for the cobalt-deficient sheep livers than for the livers from cobalt-fed, full-fed sheep. It is of interest to note, however, that the livers from the cobalt-fed, limited-fed animals also contained a much higher alkali stable vitamin B_{12} activity than the livers of the full-fed animals.

The fact that the levels of nicotinic acid and riboflavin per gram of dry rumen material are essentially the same for cobalt-fed and cobalt-deficient sheep suggests that rumen synthesis, at least for these two vitamins, was not impaired by the lack of cobalt. It should be remembered, however, that cobalt-deficient animals are at a much lower nutritional plane and the rumen-fill is much less than in a normal animal. It is not surprising, then, to find the blood levels of certain vitamins lower in these animals.

SUMMARY

Microbiological assays with *Lactobacillus leichmannii* for the vitamin B_{12} activity of the blood, livers, and rumen ingesta of cobalt-deficient sheep, cobalt-fed, full-fed sheep, and cobalt-

fed, limited-fed sheep have been presented. The blood of sheep early in cobalt deficiency contained only about one-fifth the vitamin B_{12} activity of the blood from cobalt-fed, full-fed sheep. Liver storage of vitamin B_{12} was very low in the cobalt-deficient sheep; about 30 times more "true" vitamin B_{12} activity was present in the livers of cobalt-fed animals. The alkali stable vitamin B_{12} activity of liver seemed to be higher for the deficient sheep and the cobalt-fed, limited-fed sheep than for the cobalt-fed, full-fed sheep. Rumen synthesis of vitamin B_{12} in the cobalt-deficient animals was about one-fifteenth of that encountered in the sheep given supplemental cobalt. Microbiological assays indicated that the rumen synthesis of nicotinic acid and riboflavin was essentially the same per gram of dry rumen material in deficient and cobalt-fed animals.

LITERATURE CITED

ABELSON, P. H., AND H. H. DARBY 1949 The synthesis of vitamin B_{12} in the digestive system of the sheep. Science, *110:* 566.

BROQUIST, H. P., E. L. R. STOKSTAD AND T. H. JUKES 1951 Further observations on the microbiological assay for vitamin B_{12} using *Lactobacillus leichmannii*. Proc. Soc. Exp. Biol. Med., *76:* 806.

COUCH, J. R., O. OLCESE, P. W. WITTEN AND R. W. COLBY 1950 Vitamin B_{12} content of blood from various species. Am. J. Physiol., *163:* 77.

GALL, L. S., S. E. SMITH, D. E. BECKER, C. N. STARK AND J. K. LOOSLI 1949 Rumen bacteria in cobalt-deficient sheep. Science, *109:* 468.

HALE, W. H., A. L. POPE, P. H. PHILLIPS AND G. BOHSTEDT 1950a The B-vitamins in relation to a cobalt deficiency in sheep. J. Animal Sci., *9:* 484.

———— 1950b The effect of cobalt on the synthesis of vitamin B_{12} in the rumen of sheep. Ibid., *9:* 414.

HOEKSTRA, W. G., A. L. POPE AND P. H. PHILLIPS 1952 Response of cobalt-deficient sheep to intravenously administered vitamin B_{12}. J. Nutrition, *48:* 431.

HOFFMAN, C. E., E. L. R. STOKSTAD, B. L. HUTCHINGS, A. C. DORNBUSH AND T. H. JUKES 1949 The microbiological assay of vitamin B_{12} with *Lactobacillus leichmannii*. J. Biol. Chem., *181:* 635.

KEENER, H. A., G. P. PERCIVAL, G. H. ELLIS AND K. C. BEESON 1950 A study of the function of cobalt in the nutrition of sheep. J. Animal Sci., *9:* 404.

KOCH, B. A., AND S. E. SMITH 1951 Vitamin B_{12} vs. vitamin B_{12b} for cobalt deficient sheep. Ibid., *10:* 1017.

LEWIS, U. J., U. D. REGISTER AND C. A. ELVEHJEM 1949 Vitamin B_{12} content of various organs and tissues of the rat. Proc. Soc. Exp. Biol. Med., *71:* 509.

LEWIS, U. J., D. V. TAPPAN AND C. A. ELVEHJEM 1952a A new and biologically different form of vitamin B_{12}. J. Biol. Chem., *194:* 539.

———— 1952b Distribution and some properties of vitamin B_{12f}. Abst. of 121st meeting of Am. Chem. Soc., Milwaukee, Wisconsin, 11C.

MARSTON, H. R. 1952 Cobalt, copper, and molybdenum in the nutrition of animals and plants. Physiol. Rev., *32:* 66.

PFIFFNER, J. J., D. G. CALKINS, R. C. PETERSON, O. D. BIRD, V. MCGLOHON AND R. W. STIPEK 1951 Crystalline, pseudovitamin B_{12}. Abst. of 120th meeting of Am. Chem. Soc., New York, N. Y., 23C.

RAY, S. N., W. C. WEIR, A. L. POPE AND P. H. PHILLIPS 1947 Studies on the concentration of some B-vitamins in the blood of normal and cobalt deficient sheep. J. Nutrition, *34:* 595.

SCHEID, H. E., M. M. ANDREWS AND B. S. SCHWEIGERT 1951a Liver storage of vitamin B_{12} by the rat. Proc. Soc. Exp. Biol. Med., *78:* 558.

SCHEID, H. E., AND B. S. SCHWEIGERT 1951b Liberation and microbiological assay of vitamin B_{12} in animal tissues. J. Biol. Chem., *193:* 299.

SCHWEIGERT, B. S., H. E. SCHEID AND M. M. MARQUETTE 1951 Determination of vitamin B_{12} in animal tissues. Fed. Proc., *10:* 394.

SMITH, S. E., B. A. KOCH AND K. L. TURK 1951 The response of cobalt-deficient lambs to liver extract and vitamin B_{12}. J. Nutrition, *44:* 455.

SNELL, E. E., IN P. GYÖRGY 1950 Vitamin Methods. Academic Press, Inc., New York, N. Y., vol. I.

THOMPSON, H. T., L. S. DIETRICH AND C. A. ELVEHJEM 1950 The use of *Lactobacillus leichmannii* in the estimation of vitamin B_{12} activity. J. Biol. Chem., *184:* 175.

RESPONSE OF COBALT-DEFICIENT SHEEP TO INTRAVENOUSLY ADMINISTERED VITAMIN B_{12}[1]

W. G. HOEKSTRA, A. L. POPE AND P. H. PHILLIPS

Departments of Biochemistry and Animal Husbandry, College of Agriculture, University of Wisconsin, Madison

(Received for publication July 21, 1952)

THREE FIGURES

While cobalt deficiency symptoms in sheep have been readily alleviated by oral administration of as little as 0.1 mg of cobalt per day, injection of similar or much larger amounts of cobalt have produced at most very slow responses (Becker et al., '49; Keener et al., '50; Marston and Lee, '49; Phillipson and Mitchell, '50; Ray et al., '48). This suggested that the action of cobalt was through the rumen. Keener et al. ('51) have shown that if radioactive cobalt is injected in relatively large amounts, it can pass into the rumen in significant amounts, thereby explaining the slow response of cobalt-deficient sheep to the injection of cobalt.

Filmer ('33) and Filmer and Underwood ('37) recognized the curative effects of liver on cobalt deficiency in sheep. The ash from an equivalent amount of liver would not alleviate the deficiency symptoms. They suggested that the potency of liver was due to the presence of a stored organic factor, and that cobalt functioned through the production of this factor in the body (probably via the rumen). With the discovery that vitamin B_{12} contained cobalt (Rickes et al., '48; Smith, '48), the immediate postulation was that

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

cobalt functioned through incorporation into vitamin B_{12} in the rumen. Early testing of this hypothesis led several groups of workers to conclude that the physiological role of cobalt in sheep was by some other route than through vitamin B_{12} (Becker et al., '49; Hale et al., '50; Marston and Lee, '49). In view of recent work (Koch and Smith, '51; Marston, '52; Smith et al., '51) it is apparent that administration of sufficient amounts of vitamin B_{12} will produce a definite response in the cobalt-deficient sheep.

The purpose of this paper is to present the results of intravenous administration of vitamin B_{12} to cobalt-deficient sheep, showing conclusively that vitamin B_{12} alleviates the cobalt deficiency syndrome.

EXPERIMENTAL

In December of 1950 a group of spring lambs, after parasite treatment, were fed the cobalt-deficient ration described previously (Hoekstra et al., '52). The components of the ration were mixed together and full-fed the experimental animals. Six lambs were given supplemental cobalt by mixing 1 ounce of $CoSO_4 \cdot 7H_2O$ in each 100 lb. of salt. Records of weight gains, blood hemoglobin concentrations, and feed consumption were kept. When two lambs on the cobalt-deficient ration had lost about 25% of their body weight and had a blood hemoglobin concentration around 7 gm/100 ml or lower, they were isolated and fed individually. Following a short stabilization period, one of the animals was given 20 μg of vitamin B_{12} twice daily for 21 days by intravenous administration (total dose of 840 μg). The quantity of cobalt in this amount of vitamin B_{12} is far below that needed to get recovery by cobalt injection. An isotonic solution prepared from a 0.1% triturate of crystalline vitamin B_{12} in NaCl [2] was used. The solution was sterilized by heating in boiling water for 5 minutes and was subsequently stored under refrigeration during the 21-day period of treatment. Following the first

[2] Kindly supplied by Merck and Co., Inc., Rahway, New Jersey.

treatment period, the untreated animal was made a member of another pair of experimental sheep treated in an identical manner. A total of three pairs of animals was used. Feed consumption, body weight and hemoglobin values were closely followed. Vitamin B_{12} assays were made on the blood of two sheep before treatment and at intervals during the treatment period to determine if the vitamin B_{12} injections were increasing the blood vitamin B_{12} concentrations to "near normal" values. The blood samples were taken immediately preceding the first injection of a given day, 15 hours following the previous injection.

The treated animals were continued on the cobalt-deficient ration after vitamin B_{12} injections were discontinued. They were then allowed to develop a second cobalt deficiency, whereupon they were again given intravenous injections of vitamin B_{12}, this time in doses of 40 μg once a day for 21 days. One of the sheep died of pneumonia before the second series of injections was initiated. During the depletion period one of the animals was given 3 mg of folic acid per day intramuscularly for a period of 16 days to determine if this factor had any hematological effect. The two sheep receiving the second series of vitamin B_{12} injections were slaughtered 54 days after initiation of the second treatment. The blood, livers and rumen contents of these animals were analyzed for vitamin B_{12} as described previously (Hoekstra et al., '52).

RESULTS AND DISCUSSION

Figures 1, 2 and 3 present the body weight, blood hemoglobin, and feed consumption data for the three sheep given vitamin B_{12} injections. The first series of treatments were given in sequence, sheep No. 2 serving as a negative control during the injection period for sheep No. 1 and likewise, sheep No. 3 serving as a negative control for sheep No. 2. A negative control animal for sheep No. 3 died after losing 16 lb. during the period of 43 days following initiation of injections into experimental animal No. 3. From the figures it is evident that all three animals were in a severely cobalt-deficient

state at the time of the first treatment. Each had shown weight loss (about 20 lb.), depressed appetite, and a reduced blood hemoglobin concentration. In each case the intravenous injection of 20 μg of vitamin B_{12} twice a day brought about a

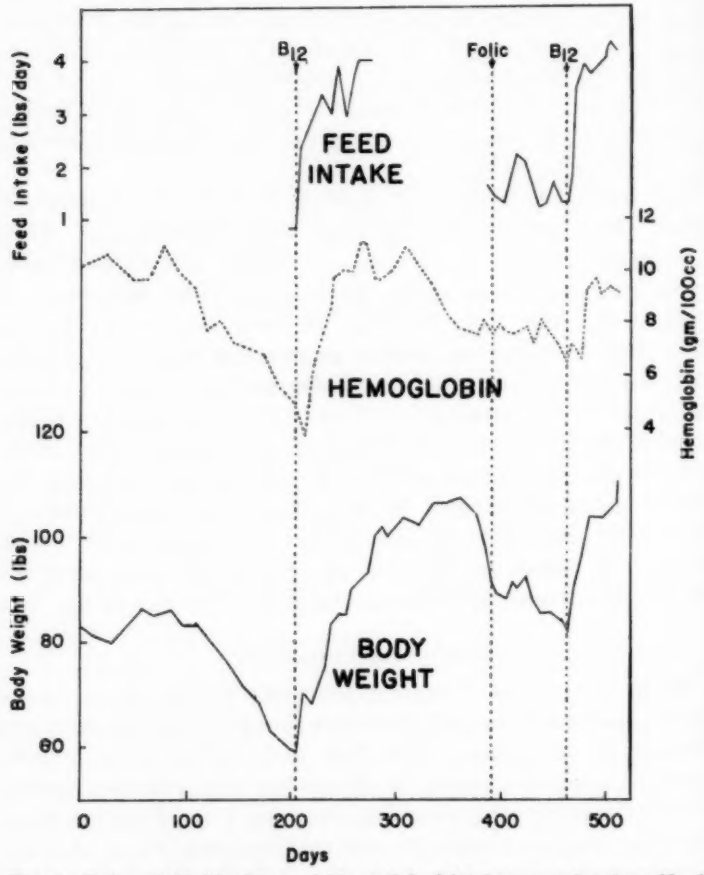

Fig. 1 Body weight, blood hemoglobin, and feed intake curves for sheep No. 1. The treatments consisted of intravenous injection of 20 μg of vitamin B_{12} twice per day for 21 days (initiated at 206 days); intramuscular injection of 3 mg of folic acid per day for 16 days (initiated at 390 days); and intravenous injection of 40 μg of vitamin B_{12} per day for 21 days (initiated at 463 days).

definite response. A remarkable increase in appetite (daily feed consumption tripled within one week) and a corresponding rapid and immediate weight gain were the first beneficial effects observed. During 80 days following initiation of treatments the three animals gained an average of 0.5 lb.

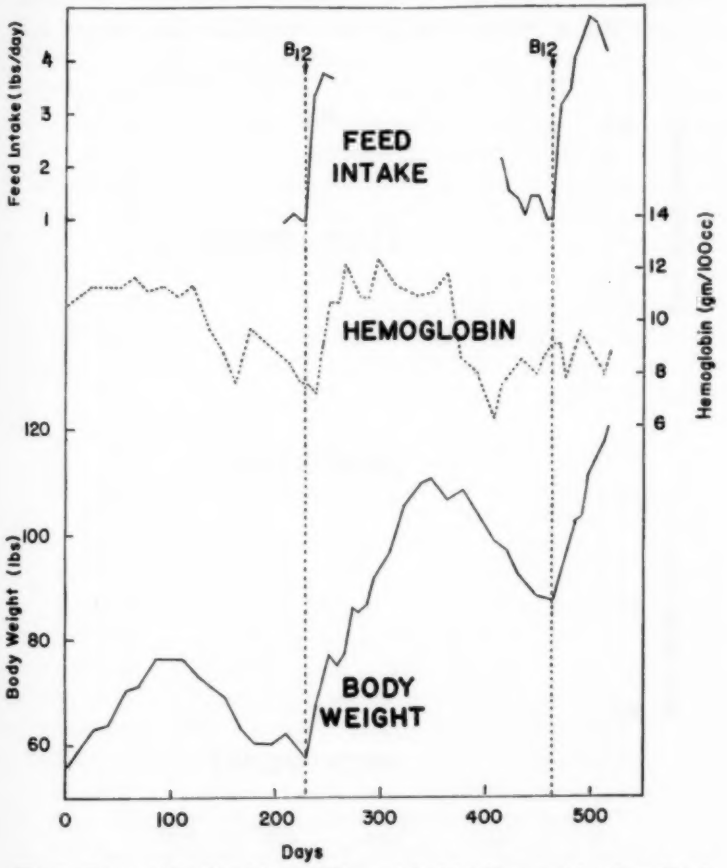

Fig. 2 Body weight, blood hemoglobin, and feed intake curves for sheep No. 2. The treatments consisted of intravenous injection of 20 μg of vitamin B_{12} twice per day for 21 days (initiated at 228 days) and 40 μg of vitamin B_{12} per day for 21 days (initiated at 463 days).

per day. The change from a dull, listless, and moribund animal to a bright-eyed, more vigorous animal was apparent within the first week. Hemoglobin responses were somewhat delayed, but in all instances returned to a normal value of 11 gm/100 ml or above within 40–60 days following initiation of vitamin B_{12} injections. A very profound response occurred in sheep No. 1, whose hemoglobin concentration rose from

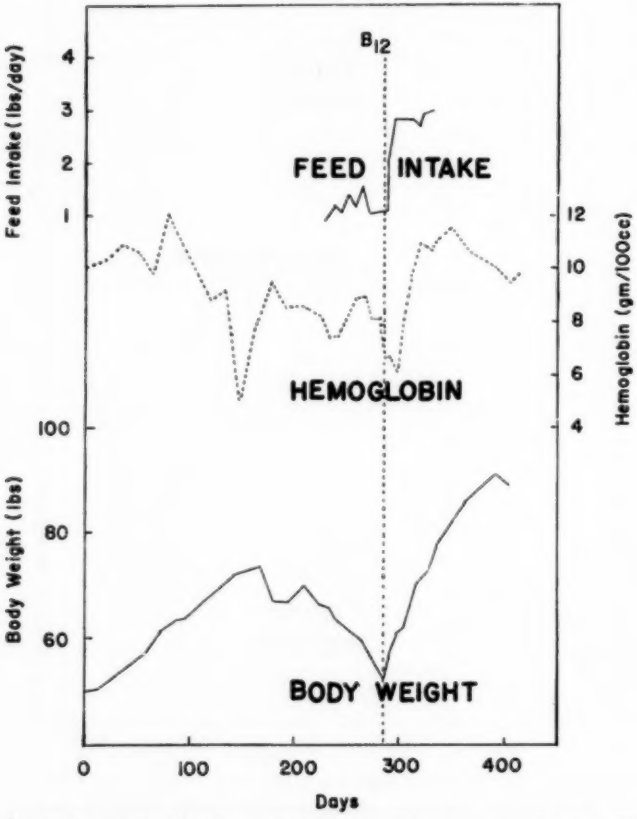

Fig. 3 Body weight, blood hemoglobin, and feed intake curves for sheep No. 3. The treatment consisted of intravenous injection of 20 μg of vitamin B_{12} twice per day for 21 days.

a low of less than 4 gm/100 ml to above 11 gm/100 ml following vitamin B_{12} administration. These observations show that vitamin B_{12} was capable of producing a definite hemoglobin response in the cobalt-deficient sheep. Numerous investigators have reported delayed and erratic hemoglobin responses after administration of cobalt to cobalt-deficient sheep (Becker and Smith, '51; Filmer, '33; Filmer and Underwood, '37; Ray et al., '48). Koch and Smith ('51) and Smith et al. ('51) have observed a similar erratic hemoglobin response following administration of vitamin B_{12} to cobalt-deficient sheep. Sheep No. 2 did not respond significantly in blood hemoglobin concentration within 54 days following the second series of vitamin B_{12} injections. The reason for these delayed and erratic hemoglobin responses in certain instances is not clear. It may indicate the lack of some other hemopoietic factor, or possibly complicating factors such as internal parasites. Spontaneous partial remissions in the hemoglobin curves as observed for sheep Nos. 2 and 3 at about 150 days are often observed in cobalt-deficient sheep when hemoglobin values are closely followed.

The amount of vitamin B_{12} administered was based upon that amount estimated to be required to maintain normal blood concentrations. Vitamin B_{12} analyses of blood samples from animals 1 and 2 before and at intervals during the injection period showed that the concentrations of vitamin B_{12} in the blood of each animal rose from about 0.4 mug/ml to between 1.0 and 2.0 mug/ml of whole blood. In another publication (Hoekstra et al., '52) we have presented the average blood vitamin B_{12} concentration of 16 sheep on the experimental ration early in cobalt deficiency as 0.47 mug/ml and that of 6 sheep full-fed the same ration plus supplemental cobalt as 2.3 mug/ml. Thus, this selected dosage of vitamin B_{12} had raised the blood vitamin B_{12} to "near normal" values. No deficient animals were fed cobalt for a direct comparison of a cobalt response with the intravenous vitamin B_{12} response observed in this study. However, the 6 animals on the same ration given supplemental cobalt from the beginning of the

experiment had gained at 210 days an average of 54 lb. (0.26 lb./day). Their hemoglobin levels were normal. This demonstrated that cobalt was definitely the limiting factor in this ration. The responses attained with vitamin B_{12} in this experiment are as good as or better than responses reported for cobalt supplementation to deficient sheep (Becker and Smith, '51; Smith et al., '51; Ray et al., '48; and many others). Koch and Smith ('51) have demonstrated equivalent responses of cobalt-deficient sheep to injected vitamin B_{12} and orally administered cobalt.

The beneficial effects of the vitamin B_{12} administrations on body weight gains continued for about 100 days following initiation of the treatment. At this time the weight curves leveled off, and soon the animals were losing weight and blood hemoglobin concentrations were falling. Animal No. 3 died of pneumonia at this time.

The administration of folic acid to sheep No. 1 is more or less incidental to this experiment. The well-known response to folic acid in pernicious anemia in the human, which is apparently a result of failure to absorb vitamin B_{12}, suggested the possibility that folic acid given in large amounts might have some effect on the hemopoietic status of the cobalt-deficient sheep. Sheep No. 2 showed little or no response to the folic acid. However, conclusions should not be made on the basis of this one animal. Studies on the relationships of other hemopoietic factors including folic acid to vitamin B_{12} in the cobalt-deficient sheep are in progress.

The second series of vitamin B_{12} injections to sheep Nos. 2 and 3 after developing a second cobalt deficiency again produced dramatic responses in weight gains and feed consumption. The hemoglobin response was suboptimal at 54 days after initiation of the treatment in sheep No. 1, and was absent in sheep No. 2. These two animals were slaughtered at this time. The results of assays for vitamin B_{12} in the blood, livers, and rumen contents of these animals are shown in table 1. The vitamin B_{12} values for cobalt-deficient sheep on the experimental ration, reported in another publication

(Hoekstra et al., '52), were approximately 0.5 mug vitamin B_{12} activity/ml whole blood, 0.03 μg "true" vitamin B_{12} activity/gm moist liver (total activity minus alkali stable activity), and 0.09 μg vitamin B_{12} activity/gm dry rumen contents. The vitamin B_{12} injected sheep showed slightly higher vitamin B_{12} concentrations in the blood, about three times as much "true" vitamin B_{12} activity in the liver, but the same amount in the rumen ingesta. These two animals were gaining weight and consuming normal amounts of feed at the time of slaughter. If the total response to the second vitamin B_{12} treatment is of the same magnitude as the response to the first treatment, and if the greater body weights of the animals at this time are considered, the animals could

TABLE 1

Vitamin B_{12} concentrations in the blood, livers and rumen contents of sheep nos. 1 and 2 at the time of slaughter

SHEEP NO.	VITAMIN B_{12} ACTIVITY OF WHOLE BLOOD	TOTAL VITAMIN B_{12} ACTIVITY OF MOIST LIVER	ALKALI STABLE VITAMIN B_{12} ACTIVITY OF MOIST LIVER	VITAMIN B_{12} ACTIVITY OF RUMEN CONTENTS
	mμg/ml	*μg/gm*	*μg/gm*	*μg/gm dry wt.*
1	0.64	0.106	0.007	0.08
2	0.61	0.073	0.007	0.10

not have been far from the limit of response to the second vitamin B_{12} treatment. This is substantiated by the blood and liver analyses. The liver vitamin B_{12} values also suggest that the duration of response to vitamin B_{12} treatment probably results from a storage of this factor during the treatment period.

This experiment represents a period in excess of 500 days during which sheep consumed a cobalt-deficient ration. The remarkable responses of the deficient animals to the initial vitamin B_{12} treatment and the similar response in a second deficiency suggests that vitamin B_{12} is by far the most important, if not the only, limiting factor in a cobalt deficiency in sheep. The erratic hemoglobin responses found after the second vitamin B_{12} treatment and those reported by Cornell workers

(Koch and Smith, '51; Smith et al., '51) to vitamin B_{12} treatment do not discredit this conclusion, since similar responses have been widely reported on administration of cobalt to cobalt-deficient sheep.

SUMMARY

Three sheep in a severe cobalt-deficiency as characterized by anemia, body weight loss, lack of appetite, and general moribund condition, were given intravenous injections of 20 μg of vitamin B_{12} twice per day for 21 days. In all of the cases this treatment was followed by an immediate and remarkable response in feed consumption, vigor, and body weight gain. Blood hemoglobin concentrations rose to normal within 40 to 60 days. The response to treatment continued for about 100 days; then the animals began developing evidence of a second cobalt-deficiency. Two of the sheep were given a second series of vitamin B_{12} injections (40 μg/day for 21 days), and again exhibited similar responses although the hemoglobin responses were somewhat erratic. The fact that these injections of vitamin B_{12} brought about such profound responses to sheep fed a cobalt-deficient ration for more than 500 days suggests that vitamin B_{12} is by far the most important, if not the only, limiting factor in the cobalt-deficient sheep.

LITERATURE CITED

Becker, D. E., and S. E. Smith 1951 The metabolism of cobalt in lambs. J. Nutrition, *43:* 87.

Becker, D. E., S. E. Smith and J. K. Loosli 1949 Vitamin B_{12} and cobalt deficiency in sheep. Science, *110:* 71.

Filmer, J. F. 1933 Enzootic marasmus of cattle and sheep. Austral. Vet. J., *9:* 163.

Filmer, J. F., and E. J. Underwood 1937 Enzootic marasmus. Austral. Vet. J., *13:* 57.

Hale, W. H., A. L. Pope, P. H. Phillips and G. Bohstedt 1950 The B-vitamins in relation to a cobalt deficiency in sheep. J. Animal Sci., *9:* 484.

Hoekstra, W. G., A. L. Pope and P. H. Phillips 1952 Synthesis of certain B-vitamins in the cobalt deficient sheep with special reference to vitamin B_{12}. J. Nutrition, *48:* 431.

Keener, H. A., R. R. Baldwin and G. P. Percival 1951 Cobalt metabolism studies with sheep. J. Animal Sci., *10:* 428.

KEENER, H. A., G. P. PERCIVAL, G. H. ELLIS AND K. C. BEESON 1950 A study of the function of cobalt in the nutrition of sheep. Ibid., *9:* 404.

KOCH, B. A., AND S. E. SMITH 1951 Vitamin B_{12} vs. vitamin B_{12b} for cobalt deficient sheep. Ibid., *10:* 1017.

MARSTON, H. R. 1952 Cobalt, copper, and molybdenum in the nutrition of animals and plants. Physiol. Rev., *32:* 66.

MARSTON, H. R., AND H. J. LEE 1949 Primary site of the action of cobalt in ruminants. Nature, *164:* 529.

PHILLIPSON, A. T., AND R. L. MITCHELL 1950 Experimental cobalt deficiency in the sheep. Brit. J. Nutrition, *4:* iii.

RAY, S. N., W. C. WEIR, A. L. POPE, G. BOHSTEDT AND P. H. PHILLIPS 1948 Studies on the role of cobalt in sheep nutrition. J. Animal Sci., *7:* 3.

RICKES, E. L., N. G. BRINK, F. R. KONIUSZY, T. R. WOOD AND K. FOLKERS 1948 Vitamin B_{12}, a cobalt complex. Science, *108:* 134.

SMITH, E. L. 1948 Presence of cobalt in the antipernicious anemia factor. Nature, *162:* 144.

SMITH, S. E., B. A. KOCH AND K. L. TURK 1951 The response of cobalt-deficient lambs to liver extract and vitamin B_{12}. J. Nutrition, *44:* 455.

THE UTILIZATION OF CALCIUM FROM LACTATE, GLUCONATE, SULFATE AND CARBONATE SALTS BY YOUNG COLLEGE WOMEN [1, 2]

MARY BROWN PATTON AND T. S. SUTTON

Departments of Home Economics and Animal Science, The Ohio Agricultural Experiment Station, Wooster

ONE FIGURE

(Received for publication July 17, 1952)

Calcium salts are often used as a source of calcium for comparison in experiments designed to study utilization of calcium from different foods. It is also a common practice for the physician to recommend the use of tablets of calcium salts to supplement that from food sources at times when large amounts are needed, such as during pregnancy and lactation. It has been found in studies of the dietary habits of older persons that many of them consume diets inadequate in calcium, and if they have not been accustomed to drinking milk, it is difficult to get them to increase their calcium intake by this means. Calcium salts have been used in such cases. In order to improve diets of the general population, particularly under emergencies such as war conditions, calcium salts have been used in the enrichment of breadstuffs and meat products. Under still other conditions, due to unequal distribution or inadequacies of food supplies, calcium salts have been recommended as a means of increasing calcium intakes. Hence it seemed worthwhile to compare the utilization of calcium from some of the salts that are used as supplements.

[1] This paper is condensed from the dissertation submitted by Mary Brown Patton in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Foods and Nutrition in the Graduate School of The Ohio State University.

[2] Journal Article 35–52, Ohio Agricultural Experiment Station.

Studies in which salts have been the sole source of calcium in the diet have been limited almost entirely to those with experimental animals since it is very difficult to obtain permission of human beings to subsist on a purified diet for a sufficient length of time to obtain reliable results. However, in one study three human subjects were maintained for 7 weeks on a purified diet in which the calcium was furnished by a mixture of phosphate and carbonate salts at a level of 879 mg of calcium daily. A natural diet supplied 789 mg daily for a three-week period (Hathaway, '49). The calcium from food sources was better utilized than that from the salts in all three cases.

Studies have been made with human beings in which the calcium in a diet of natural foods has been supplemented by that from various salts. The purpose of most of these studies has been to compare the utilization of calcium from food sources rather than that from the different salts. Two studies made among young children may be cited in which the generalization was reached that calcium from salts is retained as well as that from milk (Kempster et al., '40; Stearns and Jeans, '34). Several studies made among adults may be cited in which it was shown that calcium in salts was utilized to approximately the same extent as that in milk (Drake et al., '49; McQuarrie et al., '47; Schroeder et al., '46; Steggerda and Mitchell, '39, '46). In another study (McCance and Widdowson, '42) the conclusion was reached that the form of the salt used in bread as enrichment was not important, but that the type of flour was a factor due to the amount of phytic acid present.

PROCEDURE

The young women selected as subjects had been college students for at least one year, had no strong dislikes or allergies for foods in the diet, and all were considered healthy by the examining physician.[3]

[3] Dr. Keith Frankhauser, medical officer in charge of a Nutrition Unit of the U. S. Public Health Service stationed in Ohio in 1948–1950.

The diet differed from day to day but remained the same from week to week. Each individual food was weighed for each subject although certain ones may have been combined and served. Aliquots of a weekly food composite were dried in an oven at 60°C. and samples were burned in an oxy-calorimeter. The diet contained an average of 2179 cal. daily according to this method of determination (Fuqua, '48). Those subjects who required additional calories were given weighed amounts of foods of low calcium content. The fact that all of these young women maintained their weight within the expected fluctuations of slight weekly gains and losses indicates that the diet was probably adequate in calories.

The protein content of the diet was determined by the method of the Association of Official Agricultural Chemists ('45); the calcium by the Stearns ('29) modification of the McCrudden method; and the phosphorus by the Fiske and Subbarow ('25) molybdate method. The diet supplied an average of 57 gm protein, 347 mg calcium, and 758 mg phosphorus daily.

The ascorbic acid, thiamine, and riboflavin contents of the diet were calculated from food tables. The diet was estimated to be adequate in all respects except riboflavin and calcium. To bring the riboflavin intake to the recommended level each subject received one milligram of riboflavin daily in tablet form. The basal diet, containing 347 mg of calcium per day, was supplemented by 400 mg of calcium daily from the salts which were added in approximately equal portions at the beginning of each meal. The total of approximately 700 mg was the amount considered to be just below the critical level, that level above which an additional dietary increment would not be expected to result in additional storage but have a retention value of zero. As recommended by the Food and Nutrition Board of the National Research Council ('48), the diet was further supplemented with 500 I.U. of vitamin D in the form of "Viosterol" given daily at breakfast time.

Excreta were collected according to the customary procedures. Carmine was used as a fecal marker. Calcium was

precipitated from aliquots of an acidified composite of urine. Aliquots of feces and food composites were wet ashed and calcium determined by a modification of the McCrudden method as described by Stearns ('29).

TABLE 1

Plan of study of the utilization of 4 calcium salts

PERIOD	LENGTH OF PERIOD	SUBJECTS				
	day					
I	2–7	I No supplement	II No supplement	III No supplement	IV No supplement	V No supplement
I	2–7	VI No supplement	VII No supplement	VIII No supplement	IX No supplement	
II	2–7	I Lactate	II Sulfate	III Gluconate	IV Carbonate	V Gluconate
II	2–7	VI Sulfate	VII Lactate	VIII Carbonate	IX Gluconate	
III	2–7	I Carbonate	II Lactate	III Sulfate	IV Gluconate	V Carbonate
III	2–7	VI Gluconate	VII Sulfate	VIII Lactate	IX Carbonate	
IV	2–7	I Gluconate	II Carbonate	III Lactate	IV Sulfate	V Lactate
IV	2–7	VI Carbonate	VII Gluconate	VIII Sulfate	IX Lactate	

Serum-calcium levels were followed for each subject on blood samples collected at the beginning of the study and at the close of each experimental period. Determinations were made in the laboratory of the Ohio Department of Health.[4]

The Statistical Laboratory of The Ohio State University planned an experimental design (table 1) in which 8 subjects (Subjects I to VIII, inclusive) were to be studied. A 9th subject was included and she received the supplements indicated in table 1.

[4] These determinations were made by E. C. Tabor, chief chemist with a Nutrition Unit of the U. S. Public Health Service stationed in Ohio in 1948–1950.

RESULTS AND DISCUSSION

The calcium balances of 9 subjects during periods II, III, and IV are reported in table 2. A statistical analysis of the data for Subjects I to VIII, inclusive, was made by the method of least squares and no significant effects of salt, individual, or time (order in which the various salts were taken) could be found. If it is assumed that significant differences do exist, the question arises as to why these data fail to reveal such differences. These suggestions are offered: too few subjects, sensitivity of statistical method, and individual differences offset treatment differences.

TABLE 2

Calcium balances of 9 young women during three experimental periods when a low calcium diet was supplemented by 400 mg of calcium from salts

PERIOD	SUBJECT								
	I	II	III	IV	V	VI	VII	VIII	IX
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
II	24	70	76	47	17	32	— 25	53	73
III	— 90	— 13	65	18	— 48	10	6	88	— 1
IV	— 4	— 48	— 61	— 27	— 83	— 36	157	18	— 60

Among the differences in expected and observed values of the 24 balances of these 8 subjects, the two largest were associated with Subject VII. Observation of the values in table 2 reveals that this subject did show unusual variation, particularly as far as the time element was concerned. Inspection of the fundamental data shows that she was in negative balance during the first experimental period while all the other subjects were in positive balance. During the third experimental period this subject showed a comparatively large positive balance and all other subjects, except one, were in negative balance.

If Subject VII is replaced by Subject IX, who was observed at the same time, the symmetry in treatments will be lost. However, if it is assumed that the treatment effects are zero, an analysis of these new data may be made to see if the

statistical method used would then reveal any significance in time or in individual subject differences. When this replacement was made the mean retention on the sulfate treatment was increased from 27.33 to 31.60 mg; the mean for the lactate treatment was reduced from — 11.67 to — 17.50 mg; the mean for carbonate treatment was increased from — 20.30 to — 17.57 mg; and the mean for the gluconate treatment was decreased from 45.67 to 31.67 mg.

Based on the values when Subject IX is substituted for Subject VII, it was shown by analysis that the individual differences in the subjects cannot be considered as significant but that time produced a difference in calcium balances among these subjects that is significant at the 5% level.

Further analysis of the data, using all 9 subjects, was made to determine the percentage of utilization of each salt according to the method used by Steggerda and Mitchell ('46). In 4 cases the balance on a supplement was less than that on the basal diet alone and these cases were termed zero utilization. The mean value for carbonate utilization was 10.79%; for sulfate, 20.92; for lactate, 11.45; and for gluconate, 28.79. The less soluble salts, carbonate and sulfate, were as well utilized as the more soluble ones, gluconate and lactate. Schroeder et al. ('46) reported an average of 23.65% utilization of the calcium of calcium sulfate. Average utilizations of 20 and 25% have been reported for gluconate in two studies by Steggerda and Mitchell ('39, '46). Stearns and Jeans ('34), in a study with young children, reported variable results for lactate, gluconate and carbonate but fairly consistent results with phosphates. They reasoned that the difference was due to the variation brought about in the calcium-phosphorus ratio. The senior author of the present study was unable to relate balance to calcium-phosphorus ratio in a study among a group of 18 young college women (Patton et al., '52). The values reported above for the 4 salts used in the present study are within the range reported by these other workers and within the range of that reported for milk, the food recognized by nutritionists as the best source of calcium.

In studying the percentage utilization of the salts by the 9 subjects reported herein, it was noted that 7 of the 9 subjects utilized to the largest percentage the salt taken first, regardless of which salt it was (fig. 1). The correlation coefficient of utilization and rank was 0.67 which is significant for this number of cases. The time at which a salt was taken was shown to be a factor when the data for the 8 subjects (Subject IX substituting for Subject VII) were analyzed by the method of least squares.

Blood samples were taken from each subject at the beginning of the study and every two weeks thereafter and analyzed for

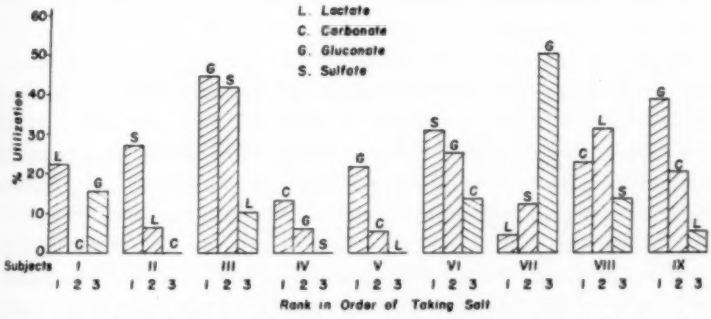

Fig. 1 Relation of the per cent utilization to the rank in order of taking a salt.

serum-calcium. The average value at the beginning was 9.8 mg % and at the end of the no-supplement period, 10.5. The levels for the periods when each of the salts was given are practically the same, 11.3, 11.5, 11.5, and 11.8, respectively, for gluconate, carbonate, lactate, and sulfate. All of the values were within the normal range of 9 to 11 mg %. The increase in blood level that was noted when 400 mg of calcium were added to their diets as salts is not in agreement with the statement by Sherman ('47) that "in normally nourished individuals, the intake of food calcium or even the administration of calcium salts alone has hardly a measurable direct influence upon the blood calcium level."

The average utilization of calcium from the three salts taken by each subject was compared with the basal heat production per square meter per 24 hours. Three subjects, whose basal metabolic rate was slightly above the Aub-DuBois predicted standard but within normal range, showed an average utilization of 22.7%. A 4th subject, whose rate was slightly below the standard but within the normal range, showed an average utilization of 21.5%. The basal metabolic rates of the remaining 5 subjects were below their predicted standard by from 7 to 27%. The average utilization of the salts by these 5 subjects was only 14.0%. These results suggest a possible relationship between calcium utilization and basal metabolic rate. Johnston and Maroney ('36) have shown that poor diets in general result in low energy metabolism. Although a record was made of the foods eaten by each subject in the study reported herein on the day previous to the beginning of the study, it has not been considered as an evaluation of their customary eating habits. This observation emphasizes the importance of noting previous dietary habits in studies such as the one reported.

The difficulty of separating feces into portions that represent those from the several periods offers a possible source of errors in studies such as this one. Two subjects, who were very regular in their habits of elimination, had formed stools in which the carmine usually appeared in from 24 to 36 hours, and the demarcation was clear cut, showed a fairly consistent utilization of calcium from the salts that supplemented their diets. One subject was constipated and the carmine usually remained in the digestive tract for from 4 to 5 days. However, this subject showed the greatest average percentage retention of calcium of any of the subjects studied. The remaining 6 subjects varied considerably as to the nature of their stools and the time of passing the carmine.

Leichsenring et al. ('51) reported that a group of 18 college women were able to adjust to the level of calcium in a basal diet similar to the one used in this study within one week. For this reason that period was used for each of the experi-

mental periods in this study. The data show that, regardless of the nature of the salt taken as a supplement, if it happened to be the first salt following the preliminary adjustment period when the intake was 300 mg, its retention was greater than that for the same or any other salt at a later period. This casts some doubt as to whether or not these 9 subjects made the adjustment from the 300 to the 700 mg level during the one week allowed for adjustment.

Another implication from these data might be that human beings do not efficiently utilize calcium from salts for periods of more than a couple of weeks. A study has been carried out in this laboratory to obtain additional information on this subject and a report of the findings is in preparation.

SUMMARY

A study was made of the utilization of calcium by 9 college women from 4 salts (gluconate, lactate, carbonate, and sulfate) given in amounts equivalent to 400 mg of calcium daily. The basal diet contained an average of 347 mg of calcium daily and calculation of other nutrients showed that the diet was adequate.

Statistical analysis of the balance data was made by the method of least squares. No significant differences in the utilization of calcium from these salts were found, the calcium from the less soluble salts being utilized as well as that from the more soluble ones.

The order in which the salts were taken was shown to be a significant factor in the utilization. In the majority of the cases, the salt taken first was utilized to the largest percentage regardless of which salt it was.

The average percentage utilization from all salts for all subjects was approximately 18%, a value within the range of those reported in the literature for utilization of calcium from food sources.

Young women having normal basal metabolic rates showed somewhat greater percentage utilization of calcium from salts than those whose rate was below the predicted normal.

The serum-calcium levels were normal, 9 to 11 mg per 100 ml, during the 8 weeks of the study and were practically the same for the periods when the 4 salts supplemented the basal diet.

LITERATURE CITED

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1945 Official and Tentative Methods of Analysis, Ed. *6*, 932 pp., illus., Washington, D. C.

DRAKE, T. G. H., S. H. JACKSON, F. F. TISDALL, W. M. JOHNSTONE AND L. M. HURST 1949 The biological availability of the calcium in bone. J. Nutrition, *37:* 369.

FISKE, C. H., AND Y. SUBBAROW 1925 The colorimetric determination of phosphorus. J. Biol. Chem., *66:* 375.

FOOD AND NUTRITION BOARD 1948 Recommended Dietary Allowances (Revised). National Research Council Reprint and Circular Series, no. 129.

FUQUA, MARY E. 1948 Comparison of the caloric value of food obtained by use of the oxy-calorimeter and food composition tables. M.S. thesis, The Ohio State University, Columbus, 41 pp.

HATHAWAY, M. 1949 Personal communication.

JOHNSTON, J. A., AND J. W. MARONEY 1936 Relationship of basal metabolism to dietary intake. Am. J. Dis. Child., *51:* 1039.

KEMPSTER, E., H. BREITER, R. MILLS, D. MCKEY, M. BERNDS AND J. OUTHOUSE 1940 The utilization of the calcium of dicalcium phosphate by children. J. Nutrition, *20:* 279.

LEICHSENRING, J. M., L. M. NORRIS, S. A. LAMISON, E. D. WILSON AND M. B. PATTON 1951 The effect of level of intake on calcium and phosphorus metabolism in college women. Ibid., *45:* 407.

MCCANCE, R. A., AND E. M. WIDDOWSON 1942 Mineral metabolism of healthy adults on white and brown bread dietaries. J. Physiol., *101:* 44.

MCQUARRIE, I., M. ZIEGLER AND I. H. MOORE 1947 Calcium enriched meat compared with milk as a source of calcium, phosphorus, and protein. Proc. Soc. Exp. Biol. Med., *65:* 120.

PATTON, M. B., E. D. WILSON AND J. M. LEICHSENRING 1952 The relation of calcium to phosphorus ratio in the utilization of these minerals among 18 young college women. In preparation.

SCHROEDER, L. J., W. M. CAHILL AND A. H. SMITH 1946 The utilization of calcium in soybean products and other calcium sources. J. Nutrition, *32:* 413.

SHERMAN, H. C. 1947 Calcium and Phosphorus in Foods and Nutrition. Columbia University Press, New York, N. Y., p. 21.

STEARNS, G. 1929 A rapid method for the preparation of fecal digests suitable for use in nitrogen and mineral analysis. J. Lab. Clin. Med., *14:* 954.

STEARNS, G., AND P. C. JEANS 1934 Utilization of calcium salts by children. Proc. Soc. Exp. Biol. Med., *32:* 428.

STEGGERDA, F. R., AND H. H. MITCHELL 1939 The calcium requirement of adult man and the utilization of the calcium in milk and in calcium gluconate. J. Nutrition, *17:* 253.

———— 1946 The effect of the citrate ion on the calcium metabolism of adult human subjects. Ibid., *31:* 423.

EFFECT OF UNTREATED CORN AND MEXICAN TORTILLA UPON THE GROWTH OF RATS ON A NIACIN-TRYPTOPHAN DEFICIENT DIET

RENÉ O. CRAVIOTO, GUILLERMO H. MASSIEU, OMAR Y. CRAVIOTO AND F. DE M. FIGUEROA

Instituto Nacional de Nutriología, Secretaría de Salubridad y Asistencia, México, D. F.

(Received for publication July 14, 1952)

Krehl et al. ('45a, b) observed that corn has a growth-depressing effect upon the rat when added to a niacin-tryptophan low diet. It has been questioned (Krehl, '49) whether that action is due to a toxic factor present in corn or is conditioned by an amino acid imbalance. Several authors (Henderson et al., '47; Krehl et al., 46a, b; Rosen and Perlzweig, '49; Singal et al., '48) have demonstrated similar effects with either amino acid mixtures, tryptophan deficient proteins or acid hydrolysates of proteins. Recently, Hankes et al. ('49), and Lyman and Elvehjem ('51) showed that mixtures of gelatin and some amino acids have the same growth-depressing action when they are fed in the presence of either L-cystine or methionine. Corn is used in México as human food largely in the form of tortillas and one of the steps in the preparation of this food is by heating it for one hour at 80°C. with lime water which is discarded after a 24-hour period; only the softened grain being ground and used (Cravioto et al., '45).

Krehl et al. ('46a) have reported that tortilla has approximately the same growth-depressing effect as untreated corn, but Laguna and Carpenter ('51) have recently reported that corn heated with lime water loses its depressing effect when

incorporated into rations similar to those used by Krehl and his co-workers. They attributed this loss to the liberation of nicotinic acid from a "precursor" when the grain was heated in lime water, *without, however,* presenting any supporting data. Furthermore, the preparation used by Laguna and Carpenter was not the same as the Mexican tortilla, since the heated grain and mother liquors were dried together and fed as such after neutralization with phosphoric acid. In the present study a comparison was made between the effect of untreated corn and Mexican tortilla upon the growth of rats on a niacin-tryptophan deficient diet, with and without supplements of cystine.

EXPERIMENTAL

A basal diet similar to that used by Krehl et al. ('45b) was prepared, but the level of casein was 11.66%, and the salt mixture of Hubbell et al. ('37) was utilized instead of the Phillips and Hart mixture. Vitamins were incorporated into the diet at the same levels used by Krehl and his co-workers. Casein (Baker's) was extracted 5 times with hot 96% ethyl alcohol and the content of nicotinic acid after this treatment was found to be less than 20 μg per 100 gm.

Six rations were prepared from the basal diet by mixing 60 parts with 40 parts of the following supplements: corn starch (ration Ia); white corn (ration IIa); air-dried tortilla, prepared according to the Mexican fashion as described by Cravioto et al. ('45) (ration IIIa); rations Ib, IIb, and IIIb were the same except for the addition of 10 mg of nicotinic acid per kilogram.

Five other rations consisted of 60 parts of the basal diet with L-cystine omitted, and 40 parts of: corn starch (ration IVa); white corn (ration Va) or air-dried tortilla (ration VIa). Ten milligrams of nicotinic acid were added per kilogram of rations IVa and Va to obtain rations IVb and Vb. The final level of casein was 7% in all rations.

Sixty-six weanling albino rats, Wistar strain, weighing 35 to 40 gm were distributed in 11 groups of 6 animals (three

males and three females) each placed individually in galvanized iron cages with wire bottoms, and each group was fed one of the rations. Food and water were given ad libitum, and the food consumption was recorded. Two drops of a corn oil solution of vitamin A (4,200 I.U./ml), vitamin D (420 I.U./ml), and α-tocopherol (0.7 mg/ml) were given to each rat weekly. During the 28-day experimental period the animals were weighed three times a week.

Nicotinic acid was determined by the U.S.P. microbiological method ('43) in the corn and tortilla as well as in a corn preparation similar to that described by Laguna and Carpenter ('51).

The results were expressed as final weights as weight gain per 100 gm of food consumed.

RESULTS AND DISCUSSION

The weight gained per 100 gm of ration Ia, which contained starch, was used as the basis for evaluating the effect of the other rations. The replacement of starch by corn resulted in a similar growth in spite of the higher level of protein in the corn ration (table 1, groups Ia and IIa). However, when starch was replaced by dried tortilla, growth was much higher than that of the animals on the corn ration (table 1, groups IIa and IIIa). Statistical analysis of the data shows that this difference is significant. The ratio of the mean difference in growth to the probable error of the difference is 13.5. The addition of 1 mg % of nicotinic acid to the corn ration produced growth equal to that obtained with the tortilla ration, but niacin did not increase growth on this latter ration.

As was expected, lower growth was obtained with ration IVa (basal + starch) from which L-cystine was omitted. It is particularly noticeable that with the omission of cystine the corn and tortilla rations produced more nearly equal rates of growth, the growth-depressing effect of corn being apparently lost. The difference in growth between groups Va and VIa is not statistically significant. It should be noted

TABLE 1

Growth of rats on the different rations studied

RATION	SUPPLEMENT	ADDED NIACIN	INITIAL WEIGHT [1]	FINAL WEIGHT [1]	FOOD CONSUMED DAILY PER RAT	WEIGHT GAINED PER 100 GM OF FOOD CONSUMED [1]
		mg %	*gm*	*gm*	*gm*	*gm*
Ia	Corn starch	..	37.9 ± 0.7	73.1 ± 2.1	7.5 ± 0.3	16.7 ± 0.7
IIa	Corn	..	36.6 ± 0.5	67.3 ± 2.5	6.0 ± 0.1	18.0 ± 0.4
IIIa	Tortilla	..	37.8 ± 0.9	107.3 ± 3.1	9.3 ± 0.3	26.5 ± 0.5
Ib	Corn starch	1	36.8 ± 0.5	63.2 ± 4.6	6.9 ± 0.5	13.5 ± 1.5
IIb	Corn	1	38.1 ± 0.9	116.1 ± 1.9	10.2 ± 0.4	26.2 ± 0.2
IIIb	Tortilla	1	37.4 ± 0.8	108.2 ± 3.5	9.3 ± 0.4	27.2 ± 0.9
IVa	Corn starch n/c [2]	..	36.8 ± 0.5	59.4 ± 1.8	6.3 ± 0.5	12.7 ± 0.6
Va	Corn n/c	..	36.4 ± 0.9	77.5 ± 4.4	6.3 ± 0.4	23.1 ± 1.1
VIa	Tortilla n/c	..	35.7 ± 0.6	95.6 ± 3.6	8.4 ± 0.4	25.5 ± 0.8
IVb	Corn starch n/c	1	36.5 ± 1.1	48.8 ± 1.5	5.5 ± 0.3	8.1 ± 0.6
Vb	Corn n/c	1	36.6 ± 0.9	98.0 ± 2.9	9.3 ± 0.1	23.6 ± 0.4

[1] Including the probable error of the mean result calculated according to the formula $\sqrt{\Sigma d^2/n} - 1/\sqrt{n} \times 0.6745$, where "d" is the deviation from the mean and "n" is the number of observations (Sherman, '41).

[2] n/c means cystine omitted (see the text).

that there is a lower gain in weight, and less food intake, in the animals fed the corn ration with cystine omitted. Therefore at least two variables may be involved in these results: one a function of food efficiency, another a function of food intake. This makes it difficult to interpret the data obtained. Nevertheless, if the groups fed diets IIa (with cystine) and Va (without cystine) are compared, it can be seen that the animals fed ration Va had a greater gain in weight than those in group IIa. It should be noted that in this case there was practically no difference in the food intake. Statistically, the difference between weight gained in groups IIa and Va is significant. The ratio of the mean difference in growth to the probable error of the difference is 4.3. It is possible that the growth-depressing effect of corn is due to an amino acid imbalance shown in the presence of cystine. This would be in agreement with the observations of Hankes et al. ('49), and Lyman and Elvehjem ('51).

The variations in food intake of the different rations make it difficult to interpret the results. It should be noted that in most of the reports by Krehl et al. ('45a,b, '46a, b) and also in the report of Laguna and Carpenter ('51) the food intake by the animals is not given. This makes it hard to compare their results with ours. The possibility exists that the differences in weight gain of the animals fed the different rations were exaggerated by the variations in food intake. Some of the groups did not eat very well, possibly because of unpalatibility of the ration due to physical texture or taste or other unknown causes. Therefore, in certain cases some degree of starvation can operate as a determining factor in protein utilization. It has been reported (Barnes et al., '45) that a restriction of the protein intake from diets low in these substances causes a decrease in their utilization.

The nicotinic acid contents of the corn and tortilla were found to be 2.33 and 2.15 mg %, respectively, as measured by microbiological assay. Liberation of more of this vitamin

could not be noted after treatment of the corn by the Laguna and Carpenter ('51) procedure.

Since no liberation of niacin was observed by treatment of the corn with hot lime water, the lack of a growth-retarding effect in tortilla cannot be explained on the basis of the Laguna and Carpenter ('51) hypothesis. It may be due to destruction or racemization of certain amino acids in the corn during the treatment with lime water. These changes appear to be most marked for threonine, arginine, and histidine (Massieu et al., '49), and they might be enough to minimize the amino acid imbalance of untreated corn. Nevertheless, because of the low intake of the corn ration, some degree of starvation may have operated as mentioned above, with less protein being made available for growth, and therefore, a lower figure being obtained for the last column. Hence this lower figure is not necessarily due to amino acid imbalance. Further study of the problem is needed in order to settle the question.

The reason for the disagreement between the growth-depressing effect of tortilla reported by Krehl et al. ('46a) and the results of Laguna and Carpenter ('51) as well as those presented in this paper might be due to differences in the treatment of the corn; but data would be necessary to find the actual reason for such a discrepancy.

SUMMARY

A study was made of the effect of corn and Mexican tortilla upon the growth of weanling rats fed diets low in niacin and tryptophan, with and without supplements of cystine.

Corn inhibited growth in cystine-supplemented rations, but tortilla actually stimulated growth. In the absence of cystine corn apparently failed to depress the growth, which was similar to that in rats fed tortilla.

ACKNOWLEDGMENT

The authors wish to thank Dr. Jesús Guzmán for his cooperation and assistance in the preparation of this paper.

LITERATURE CITED

BARNES, R. H., JEAN E. MAACK, MARY J. KNIGHTS AND G. O. BURR 1945 Measurement of the growth-promoting quality of dietary protein. Cereal Chem., *22:* 273.

CRAVIOTO, R. O., R. K. ANDERSON, F. DE P. MIRANDA AND R. S. HARRIS 1945 Nutritive value of the Mexican tortilla. Science, *102:* 91.

HANKES, L. V., L. M. HENDERSON AND C. A. ELVEHJEM 1949 Effect of cystine and threonine on the growth of rats receiving niacin-tryptophan deficient rations. J. Biol. Chem., *180:* 1027.

HENDERSON, L. M., T. DEODHAR, W. A. KREHL AND C. A. ELVEHJEM 1947 Factors affecting the growth of rats receiving niacin-tryptophan deficient diets. Ibid., *170:* 261.

HUBBELL, R. B., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. J. Nutrition, *14:* 273.

KREHL, W. A. 1949 Niacin in amino acid metabolism. Vitamins and Hormones, *7:* 111.

KREHL, W. A., L. M. HENDERSON, J. DE LA HUERGA AND C. A. ELVEHJEM 1946a Relation of amino acid imbalance to niacin-tryptophane deficiency in growing rats. J. Biol. Chem., *166:* 531.

KREHL, W. A., P. S. SARMA AND C. A. ELVEHJEM 1946b The effect of protein on the nicotinic acid and tryptophane requirement of the growing rat. Ibid., *162:* 403.

KREHL, W. A., L. J. TEPLY AND C. A. ELVEHJEM 1945a Corn as an etiological factor in the production of nicotinic acid deficiency in the rat. Science, *101:* 283.

KREHL, W. A., L. J. TEPLY, P. S. SARMA AND C. A. ELVEHJEM 1945b Growth-retarding effect of corn in nicotinic acid-low rations and its counteraction by tryptophan. Ibid., *101:* 489.

LAGUNA, J., AND K. J. CARPENTER 1951 Raw versus processed corn in niacin-deficient diets. J. Nutrition, *45:* 21.

LYMAN, R. L., AND C. A. ELVEHJEM 1951 Further studies on the amino acid imbalance produced by gelatin in rats on niacin-tryptophan low ration. Ibid., *45:* 101.

MASSIEU, H. G., J. GUZMÁN, R. O. CRAVIOTO AND J. CALVO 1949 Determination of some essential amino acids in several uncooked and cooked Mexican foodstuffs. Ibid., *38:* 293.

ROSEN, F., AND W. A. PERLZWEIG 1949 The effect of gelatin on the transformation of tryptophan to niacin in rats on low casein diets. J. Biol. Chem., *177:* 163.

SHERMAN, H. C. 1941 Chemistry of Food and Nutrition. The Macmillan Co., New York, N. Y., p. 573, 6th ed.

SINGAL, S. A., V. P. SYDENSTRICKER AND J. M. LITTLEJOHN 1948 Further studies on the effect of some amino acids on the growth and nicotinic acid storage of rats on low casein diets. J. Biol. Chem., *176:* 1063.

UNITED STATES PHARMACOPEIA 1943 First supplement. Nicotinic acid and nicotin-amide assay. Vol. XII, p. 69.

THE RELATIONSHIP BETWEEN THE PROTEIN CONTENT OF CORN AND THE NUTRITIONAL VALUE OF THE PROTEIN

H. H. MITCHELL, T. S. HAMILTON AND JESSIE R. BEADLES[1]
Division of Animal Nutrition, University of Illinois, Urbana

(Received for publication August 17, 1952)

The protein content of corn on the dry basis may be varied widely by selective breeding, by weather conditions, and by crop and soil management practices. At the Illinois Agricultural Experiment Station, starting with a variety of corn containing 10.92% protein, selection through 50 generations for high- and low-protein characteristics has produced a high-protein strain averaging 19.45% protein, and a low-protein strain averaging 4.91% (Woodworth, Leng and Jugenheimer, '52).

The effect of crop yield, soil type and treatment and seasonal conditions on the composition of corn and other grains has been studied by Stubblefield and DeTurk ('40). In an intensive investigation of the effects of soil treatment and crop rotation on the composition of corn, Hamilton, Hamilton, Johnson and Mitchell ('51) observed a 40% increase in the protein content of the same varieties of corn during the same season under the best conditions of soil and crop management over that under the worst conditions imposed (10.4% as compared with 7.5% on the dry basis).

Hopkins, Smith and East ('03) were the first to demonstrate that changes in the protein content of corn induced by selective breeding are related to changes in the proportions existing among the anatomical parts of the kernel.

[1] Deceased.

The most prominent change in the physical composition accompanying increasing protein content is a greatly increased proportion of the horny over the starchy endosperm. This anatomical change was observed by Hamilton et al. ('51) to accompany increased protein content of corn induced by changes in soil treatment and crop management. It was also shown in this investigation (1) that the horny endosperm contains a higher concentration of protein than the floury endosperm, (2) that all the major parts of the kernel contribute, though in different degrees, to the increase in the total protein of the corn kernel, and (3) that the alcohol-soluble protein, zein, is for all practical purposes confined to the endosperm, the horny endosperm holding 60 to 65% of the endosperm zein. One would therefore expect the zein content of corn to increase in percentage more rapidly than does the total protein under conditions favoring the production of high-protein corn.

The first to discover the relationship between the proportion of zein in corn protein and the nitrogen content of the whole kernel were Showalter and Carr ('22). Hansen, Brimhall and Sprague ('46) defined this relationship more precisely for 18 different corn samples covering the range 6.3 to 19.7% protein. They established a correlation coefficient of + 0.92 and a linear regression of zein content on total protein content. Frey ('51), working in the same laboratory with 90 to 100 samples of corn ranging in protein content from 4.9 to 18.4%, also observed a high correlation between the percentage of zein and the percentage of total protein. However, this author chose to describe the relation of these two variables with a simple parabolic equation of the type used by Huxley ('32) to describe the phenomenon of relative growth, i.e., $y = ax^b$, without showing that the parabolic equation gave a better fit than would a linear equation. However, in the chosen equation, the value of b, 1.87, was significantly greater than 1, testifying to the more rapid increase of zein than of total protein in the corn kernel.

The demonstration that the proportion of zein in the total protein of corn is directly correlated with the content of protein in corn logically led to studies of the amino acids in corn because of the nutritionally unbalanced character of the essential amino acids in zein. The problem was studied from the genetics standpoint by Doty et al. ('46), with results indicating that ". . . it might be possible by careful breeding to develop a hybrid with a high proportion of nutritionally essential amino acids in its protein . . ." Miller, Aurand and Flach ('50) determined lysine, methionine and tryptophan contents of corn samples varying from 8.95 to 13.02% in total protein. They observed high correlations between protein content and the contents of each of these amino acids, but, contrary to expectation, were unable to detect any differences in the distribution of these amino acids between high- and low-protein corn. The observations of Frey ('51) are in harmony with the well-established fact that high-protein corn contains a greater proportion of zein in its total protein than does low-protein corn: tryptophan, absent in zein, and valine, in which zein is relatively deficient, occurred in decreasing proportions in the total protein of corn as the protein percentage increased.

The investigations on the chemical composition of corn and its modifying factors discussed above would lead one to expect a low nutritional value of zein, an expectation that has been amply realized by the investigations of Osborne ('13) and of Osborne and Mendel ('14). In fact, later experiments (Harris, Neuberger and Sanger, '43; Geiger and Hagerty, '49; Kligler and Krehl, '50) have been interpreted to mean that zein, even when supplemented with the essential amino acids in which it is deficient, will not support satisfactory growth, due to some hypothetical impaired digestibility, or "amino acid imbalance." The chemical data on corn would also lead one to expect that the nutritional quality of the proteins of high-protein corn is lower than that of low-protein. But this has not been proved. The experiments of Dobbins et al. ('50) with growing pigs indicated the validity

of this expectation, but unfortunately the number of pigs used was small and the variability among biological values was large so that no statistical difference could be established for corn containing 10.8 and 15.8% protein.

It will be the purpose of this report to test by biological means the expectations based upon the chemical composition of high- and low-protein corn with reference to possible differences in digestibility and biological value and the supplementary effects of tryptophan and of lysine in which zein is severely deficient. Growing albino rats have been used as experimental subjects.

EXPERIMENTAL METHODS

The experimental diets were all made according to the same pattern, the general features of which may be briefly described as follows: they were all complete in minerals and vitamins; all comparable diets were equalized in fiber at 2.0 or 2.25%, in fat at 5%, and in protein at levels varying in the different experiments. The corn components, consisting of entire kernels, varied in the different experiments; the lysine supplements when used were incorporated at a level of 0.25% and the tryptophan supplements at a level either of 0.20% or 0.40%. In one growth experiment a protein supplement consisting of two parts of meat scraps, two parts of soybean oil meal and one part of alfalfa meal was added to diets containing a constant proportion of corn of variable protein content, in amounts to equalize the protein content at about 18.5%. In all cases corn starch was used to complete the diets.

The nitrogen balance studies were carried out according to the usual procedure adopted in this laboratory, the essential features of which may be described briefly. Five or 6 pairs or trios of rats were carried through a series of experimental periods, each of which consisted of a preliminary feeding period of 7 to 10 days, followed by a collection period of 7 days, with constant food consumption during the preliminary and collection periods. Also, within each pair

or trio the food consumption was equalized throughout all periods. The general order of experimental periods may be illustrated by the order of diet testing followed in experiment 386:

	PERIOD I	PERIOD II	PERIOD III	PERIOD IV	PERIOD V
No. 1 rat in each pair	852	854	853	853s	852s
No. 2 rat in each pair	853	854	852	852s	853s

Diets 852 and 853 are the unsupplemented test diets, 852s and 853s are the corresponding diets supplemented with tryptophan and lysine, while diet 854 is the standardizing diet containing 4% of whole egg protein as the sole source of nitrogen. The nitrogen balance data secured in period II permit the calculation of the body's contribution to the feces (metabolic fecal N per gram of dry matter consumed) and to the urine (endogenous urinary N per $W_{gm}^{3/4}$). These nitrogen balance items are essential for the calculation of coefficients of true digestibility (T.D.) and biological values (B.V.) of the dietary nitrogen (protein). The formulas for these terminal calculations may be written as follows:

$$\text{T.D.} = \frac{\text{N intake} - (\text{fecal N} - \text{metabolic fecal N})}{\text{N intake}} \times 100$$

$$\text{B.V.} = \frac{\text{N balance} + \text{endogenous N in urine} + \text{metabolic fecal N}}{\text{N intake} - (\text{fecal N} - \text{metabolic fecal N})} \times 100$$

The numerator in the second equation epitomizes the uses to which the truly absorbed N is put in the body of the growing rat, i.e., nitrogen retention in growth and the replacement of body nitrogen losses in urine and feces in maintenance.

In the growth experiment referred to above, the empty carcasses of the rats at the termination of the experiment were analyzed for nitrogen and gross energy.

Diets, feces and urine collections, and carcasses were analyzed for nitrogen by the Kjeldahl method, using mercury as a catalyst and digesting, after clearing, for 4 hours in the case of diets, feces and carcasses, and for one hour in the case of urines. The gross energy (heat of combustion) of the

diet and carcass samples was determined with the oxygen-bomb calorimeter.

The corn samples were analyzed for total nitrogen and for nitrogen soluble in 71% ethanol, presumably zein nitrogen. They were also analyzed for tryptophan and lysine by microbiological methods. For tryptophan, hydrolysis of the protein was effected with 6 N $Ba(OH)_2$ by a modification of the method of Miller and Ruttinger ('50). The organism employed was *Streptococcus faecalis R.*, and light transmission at 650 mμ was read in a Coleman spectrophotometer. For lysine determination, the samples were hydrolyzed with 2 N HCl for 5 hours at 15 lb. pressure. The organism employed was *Lactobacillus mesenteroides* P-60, following the procedure of Henderson and Snell ('48).

EXPERIMENTAL RESULTS

The chemical constituents in the corn samples submitted to nutritional evaluation are summarized in table 1. Particularly noteworthy are:

1. The increasing proportion of zein in the total protein as the protein content of the corn samples increases. In a much larger series of corn samples, 84, with protein contents ranging from 5.5 to 13.47%, the correlation coefficient was + 0.95, and the regression of zein percentage in total protein (y) on total protein (x) was $y = -17.44 + 5.20x$. The regression departed from linearity at higher protein contents.

2. The decreasing percentage of tryptophan in the total protein (y) as the zein content of the total protein (x) increases. In the large set of data referred to above, this correlation is measured by a coefficient of − 0.81, and a regression of $y = 1.126 - 0.0101x$.

3. The decreasing percentage of lysine in the total protein (y) as the zein content of the total protein (x) increases. For the large set of data, the correlation between these two variates is measured by a coefficient of − 0.76, and a regression of $y = 4.029 - 0.0429x$.

The correlation and regression coefficients cited above are all highly significant statistically.

The nutritional evaluation of the proteins of the corn samples tested, with and without amino acid supplements, is given in table 2, together with statistical analyses of the differences between the average biological values in the three experiments.

TABLE 1

Pertinent constituents in corn samples tested

DESCRIPTION OF SAMPLES	PROTEIN (N × 6.25) CONTENT ON DRY BASIS	ETHANOL-SOLUBLE IN TOTAL PROTEIN	TRYPTOPHAN IN TOTAL PROTEIN	LYSINE IN TOTAL PROTEIN
	%	%	%	%
U.S. Hybrid 13, continuous corn, no soil treatment	7.32	23.2	0.87	2.92
U.S. Hybrid 13, corn, oats and clover rotation; lime, manure and rock phosphate fertilization	10.73	32.9	0.75	2.72
Ill. high protein grown on nitrogen-deficient soil	13.47	46.4	0.71	2.19
Ill. high protein grown on nitrogen-fertilized soil	20.04	57.7	0.55	1.76
Funk's Hybrid G94	9.23	45.1	0.76	2.19
Ill. high protein grown on nitrogen-fertilized soil	21.07	56.5	0.57	1.77

The digestibility of the nitrogen in all experiments averaged higher for the high-protein corn. The differences in digestibility were statistically significant at probability levels of 3% or less except in the comparison of diets 859 and 858 ($P = 0.09$) and diets 852 and 853. However, the digestibility of the nitrogen in diets 858s and 859s, averaging 94.8 and 95.9, respectively, differed significantly ($P < 0.0001$). These data are not given in table 2, because the biological values obtained on the supplemented diets were so erratic as to indicate clearly the operation of gross disturbing factors in

TABLE 2

The nutritive value of the proteins measured by nitrogen balance studies with growing rats

EXP. NO.	DIET NO.	CORN SAMPLES COMPARED AND THEIR PROTEIN CONTENTS ON THE DRY BASIS	AMINO ACID OR PROTEIN SUPPLEMENTS	PROTEIN CONTENT OF DIETS	NUMBER OF PAIRS USED IN EACH EXPERIMENT	AVE. DIGESTIBILITY OF PROTEIN	AVE. BIOLOGICAL VALUES OF PROTEIN	STATISTICAL COMPARISONS OF BIOLOGICAL VALUES			
								Diets compared	Mean difference	Student's t value	Student's P value
		%		%		%	%				
386	852	7.32%, U. S. hybrid 13 [1]	None	6.24	5 in all	88.3	68.6	852 vs. 853	5.5	1.91	0.044
	853	10.73%, U. S. hybrid 13 [2]	None	6.24		92.5	63.1	852 vs. 852s	10.4	3.72	0.0024
	852s	Same as 852	0.25% DL-lysine and 0.20% L-tryptophan	6.31		88.5	79.0	853 vs. 853s	14.2	7.02	< 0.0001
	853s	Same as 853		6.56		91.7	77.3	852s vs. 853s	1.7	0.93	0.19
446	858	13.47%, Ill. high protein [3]	None	10.38	6 in all	94.8	46.9	858 vs. 859	2.1	3.68	0.0019
	859	20.04%, Ill. high protein [4]	None	10.56		95.3	44.7				
446a	864	9.23%, Funk's G94	None	8.06	6 in all	93.1	56.8	864 vs. 865	7.6	5.08	0.0002
	865	21.07%, Ill. high protein	None	7.88		95.4	49.2	864 vs. 864s	3.7	3.85	0.0014
	864s	Same as 864	0.25 DL-lysine and 0.40% DL-tryptophan	8.06		93.3	60.6	865 vs. 865s	3.8	2.13	0.028
	865s	Same as 865		7.88		95.9	53.2	864s vs. 865s	7.4	4.54	0.0005

[1] Grown on soil of low fertility, plot 3NE (Hamilton et al., '51).

[2] Grown on soil of high fertility, plot 58W (Hamilton et al., '51).

[3] Grown on a nitrogen-deficient soil.

[4] Grown on a nitrogen-fertilized soil.

metabolism, but not in digestion. The preponderance of evidence clearly supports the conclusion that increased digestibility of the nitrogen accompanies an increased protein content of corn. For this small number of paired variates (6), the correlation coefficient is $+0.82$ and the regression of digestibility on protein content is $y = 88.0 + 0.3838x$. Both the correlation coefficient and the regression coefficient, 0.3838, are significant at somewhat less than the 5% level of probability.

The biological values (y) of the protein of the various corn samples tested show an inverse relationship with the protein content (x), $r = -0.78$, $y = 73.59 - 1.372x$. However, neither r nor the regression coefficient, -1.372, is significant statistically with $P = 0.077$. It is fair to consider that the statistical inadequacy is due to fewness and variability of data rather than to a possible independence of the variates, because of the established fact that the higher the protein content of corn, the greater the proportion of zein and the smaller the proportion of tryptophan and of lysine in the total protein.

In experiment 386, supplementation of the corn protein (U.S. 13) with tryptophan and lysine boosted the biological value of both low- and high-protein samples by 10 and 14 percentage points, respectively, increases that were highly significant statistically. Furthermore, the supplemented proteins, unlike the unsupplemented, possessed biological values that are indistinguishable statistically. This finding is in harmony with that of Marais and Smuts ('40), who reported an elevation of the biological value of corn, with a protein content of about 10%, from 67 to 81 by supplementation with tryptophan and lysine. Supplementation with either of the two amino acids alone had no appreciable effect on the biological value.

In experiment 446a, supplementation of both the low- and the high-protein corn with tryptophan and lysine raised the biological value of the protein, but only by somewhat less than 4 percentage units. Also, the supplemented corns show

the same difference in biological value as the unsupplemented, 7.4 and 7.6 percentage units, respectively. It should be noted, however, that unlike the case in experiment 386, the low- and high-protein corn, besides differing in protein content, differed also in variety and possibly also in the amino acid content of their proteins other than in tryptophan and lysine.

The growth experiment, no. 438, was designed to test the growth-promoting value of the protein in three corn samples containing 9.56, 15.80 and 19.69% protein on the dry basis, when supplemented with a protein concentrate mixture commonly used in swine feeding. The corn samples were incorporated in the diets at a constant percentage, 69.20, and the protein supplement, containing 45.31% of protein, at such levels as were required to raise the protein contents of the diets to approximately 18.5%.

Eleven trios of albino rats were employed in the test, with initial body weights ranging from 55 to 65 gm. One member of each trio was fed diet 830, one member diet 831 and the remaining member diet 832. The food intake of trio mates was equalized throughout the experiment, which lasted for 42 days. At the end of the feeding test, the rats were killed and the empty carcasses analyzed for protein ($N \times 6.25$) by the Kjeldahl method and the heat of combustion was determined with the oxygen-bomb calorimeter.

The main results of this experiment are summarized in table 3, with a statistical analysis of those diet differences that were the most significant when analyzed by the method of Student ('25).

Diets 830 and 831, containing corn samples with 9.56 and 15.80% protein, respectively, did not differ significantly in growth-promoting ability, as measured by body weight gains, by attained body length from nose to root of tail, or by protein deposition. The energy gain in the carcass was distinctly greater on the diet, 831, containing the corn sample with the greater content of protein.

Diets 831 and 832, containing corn components with 15.80 and 19.69% of protein, respectively, differed significantly in

TABLE 3

Results of the growth test, experiment 438

DIET NO.	PROTEIN IN CORN TESTED [1]	CORN IN DIET	PROTEIN IN SUPPLEMENT [2]	SUPPLEMENT IN DIET	PROTEIN IN DIET	AVERAGE TOTAL BODY WEIGHT GAINS [3]	AVERAGE BODY LENGTHS	AVERAGE PROTEIN IN CARCASS	AVERAGE ENERGY IN CARCASS
	%	%	%	%	%	*gm*	*mm*	*gm*	*Cal.*
830	9.56	69.20	45.31	26.55	18.25	171.3	211.1	44.27	582
831	15.80	69.20	45.31	19.11	18.56	171.0	210.4	42.88	629
832	19.69	69.20	45.31	12.05	18.69	164.5	208.9	40.90	602
Statistical analysis of data:				diets compared		831,832	831,832	831,832	830,831
				mean difference		6.45	1.5	1.98	47.1
				Student's P value		0.027	0.031	0.0048	0.0008

[1] On the dry basis.

[2] Meat scraps 2, soybean oil meal 2, alfalfa meal 1.

[3] Computed on the final empty body weight.

growth-promoting potency in favor of diet 831, as measured by body weight gains, attained body length after 42 days on feed, and protein deposition. The deposition of energy was greater on diet 831 by 27.4 cal., significant at the 4.2% level of probability.

DISCUSSION

This investigation, in conjunction with evidence published elsewhere, proves clearly that conditions, hereditary or environmental, that increase the protein content of corn, increase to a greater extent the content of the protein of poorest nutritional value, zein. Chemical and biological assays testify to the poorer nutritional quality of the protein of high-protein than of low-protein corn. The regression of percentage of zein in the total proteins of corn on total protein is such that, on the average and for protein contents up to 14% or thereabout, an increase of 1% in the total protein of corn will increase the proportion of zein by 5.2 percentage units on the average.

Biological tests of corn samples of greatly different protein content, using growing albino rats as subjects, reveal clearly, first, that the nitrogen of high-protein corn is more digestible than that of low-protein corn, the regression being such that each increase in the protein content of 1% is associated with an average decrease in the digestion coefficient of protein of 0.38 percentage units. The tests reveal further, in harmony with the chemical data, that increasing protein content is associated with decreasing biological utilization for maintenance and growth of the absorbed nitrogen. The regression is such that an increase of 1% in protein content of corn is associated with an average decrease of 1.37 in the biological value.

However, the low biological value of the proteins of corn, and the decreasing value as the protein content increases, is not a serious matter when corn is used in animal feeding. The suggestion of plant geneticists (Doty et al., '46; Frey, Brimhall and Sprague, '49) that the situation may be corrected by selective breeding of corn seems needlessly diffi-

cult. The easiest way and the most practical way is to use corn in farm rations in combinations with feeds capable of correcting the amino acid deficiencies of corn.

The content of corn proteins in the amino acids essential for growth reveals the fact that these proteins are limited in their biological utilization by a serious deficiency of lysine and of tryptophan (Mitchell and Block, '46). In this investigation it has been shown that supplementation of corn proteins with these two amino acids may boost the biological value by 10 to 14 percentage units, confirming earlier work by Marais and Smuts ('40), raising the biological value to a level comparable to that of meat protein.

At present it is impractical to correct the amino acid deficiencies of corn by pure amino acids, but it has been known for some time that the desired result can be secured by feeding corn in combination with feed proteins relatively rich in tryptophan and lysine. One of the first demonstrations of this possibility was reported by Hart, Steenbock and Letcher ('22), who showed that corn proteins when combined with milk proteins in the proportion of 1 to 0.4 yield a protein mixture which is highly efficient in promoting nitrogen retention in growing pigs. Calculations from their data (Mitchell, '24) show that the biological value of the proteins of such a mixture is about 87, compared to 60 for corn proteins.

Dobbins et al. ('50) tested the supplementary relations for growing pigs of a mixture of equal parts by weight of alfalfa meal, soybean meal and meat and bone scraps added to rations containing corn varying in protein content from 8.2 to 12.8%. When the ratio of corn N to supplemental N remained above 1.00, the protein mixtures were equally efficient in promoting growth. Only when for the rations containing the highest protein corn the ratio fell to 1:0.57, and, for heavier body weights, to 1:0, was the inferior nutritional value of the corn containing 12.8% protein evident.

In the growth test with rats of the investigation here reported, much the same supplemental protein mixture was

used as that employed by Dobbins et al. Here it was shown that for corn containing 15.8% protein, a ratio of corn N to supplemental N of 1:1 (diet 831) was as good nutritionally, within the limitations of the technic employed, as a ratio of 1:1.85 (diet 830) with corn containing 9.56% protein. However, in diet 832, containing corn with 19.69% protein and a ratio of corn N to supplemental N of 1:0.435, a depression in growth and protein storage was revealed.

The proteins of soybeans, meat scraps and alfalfa meal are not deficient nutritionally in either tryptophan or lysine; the mixture used in this investigation contained about 1.16% tryptophan and 5.9% lysine on the crude protein content, according to calculations based on data reported by Stokes et al. ('45) and Block and Bolling ('51). Apparently when such a mixture is fed with corn of a protein content as high as 16%, so that the ratio of corn N to supplemental N does not fall much below 1:1, the nutritive quality of the mixture is not appreciably different from that resulting from a mixture of average protein corn (9.5%) and supplement in which the protein ratio is much higher, 1:1.85.

CONCLUSIONS

The proportion of zein in the total proteins of corn increases linearly with the total protein content up to a content of about 14%. At higher protein levels the regression apparently departs from linearity. Within the limits indicated the correlation of the two variates is high with $r = +0.95$.

The proportions of tryptophan and of lysine in the total proteins of corn decrease with increasing content of protein.

The digestibility of the mixed proteins of corn increases slightly as the protein content of corn increases, but the biological value decreases considerably. Supplementation of the proteins of corn with lysine and tryptophan will raise the biological value to levels that may approximate the biological value of meat protein ($N \times 6.25$).

Effective supplementation, even of the proteins of the highest-protein corn tested, may be attained with protein mixtures commonly used in swine feeding. Hence, the lowered biological value of the proteins of high-protein corn is not a detriment to their proper use in swine, and presumably poultry, feeding, and, within limits of protein content undefined at present, will spare the use of protein supplements.

ACKNOWLEDGMENTS

The authors appreciate the courtesy of Dr. E. B. Earley of the Department of Agronomy in securing the corn samples used in this investigation and in providing the necessary information concerning their hereditary background and the conditions under which they were grown.

LITERATURE CITED

Block, R. J., and D. Bolling 1951 The Amino Acid Composition of Proteins and Foods. Analytical Methods and Results. Charles C Thomas, Springfield, Ill., 2nd ed.

Dobbins, F. A., J. L. Krider, T. S. Hamilton, E. B. Earley and S. W. Terrill 1950a Comparison of high and low protein corn for growing-fattening pigs in drylot. J. Animal Sci., *9:* 625.

———— 1950b Comparison of high and low protein corn as evaluated by the biological value method. Ibid., *9:* 654. Proc., Am. Soc. Animal Production.

Doty, D. M., M. S. Bergdoll, H. A. Nash and A. M. Brunson 1946 Amino acids in corn grain from several single cross hybrids. Cereal Chem., *23:* 199.

Frey, K. J. 1951 The interrelationships of proteins and amino acids in corn. Ibid., *28:* 123.

Frey, K. J., B. Brimhall and G. F. Sprague 1949 The effects of selection upon protein quality in the corn kernel. Agron. J., *41:* 399.

Geiger, E., and E. B. Hagerty 1949 Growth of rats on hydrolyzed zein. Arch. Biochem., *21:* 239.

Hamilton, T. S., B. C. Hamilton, B. C. Johnson and H. H. Mitchell 1951 The dependence of the physical and chemical composition of the corn kernel on soil fertility and cropping system. Cereal Chem., *28:* 163.

Hansen, D. W., B. Brimhall and G. F. Sprague 1946 Relationship of zein to total protein in corn. Ibid., *23:* 329.

Harris, H. A., A. Neuberger and F. Sanger 1943 Lysine deficiency in young rats. Biochem. J., *37:* 508.

HART, E. B., AND H. STEENBOCK 1922 At what level do the proteins of milk become effective supplements to the proteins of a cereal grain? J. Biol. Chem., *42:* 167.

HENDERSON, L. M., AND E. E. SNELL 1948 A uniform medium for determination of amino acids with various microorganisms. Ibid., *172:* 15.

HOPKINS, C. G., L. H. SMITH AND E. M. EAST 1903 The structure of the corn kernel and the composition of its different parts. Ill. Agr. Exp. Sta. Bull. *87*, pp. 77–112.

HUXLEY, J. S. 1932 Problems of Relative Growth. Methuen and Co., Ltd., London.

KLIGLER, D., AND W. A. KREHL 1950 Lysine deficiency in rats. I. Studies with zein diets. J. Nutrition, *41:* 215.

MARAIS, J. S. C., AND D. B. SMUTS 1940 The biological value of the proteins of maize and maize supplemented with lysine and tryptophane. Onderstepoort J. Vet. Med., *15:* 197.

MILLER, R. C., L. W. AURAND AND W. R. FLACH 1950 Amino acids in high and low protein corn. Science, *112:* 57.

MILLER, S., AND V. RUTTINGER 1950 An improved method of hydrolysis for use in the microbiological determination of tryptophan in human milk. Arch. Biochem., *27:* 185.

MITCHELL, H. H. 1924 The nutritive value of proteins. Physiol. Rev., *4:* 424.

MITCHELL, H. H., AND R. J. BLOCK 1946 Some relationships between the amino acid contents of proteins and their nutritive values for the rat. J. Biol. Chem., *163:* 599.

OSBORNE, T. B. 1913 The nutritive value of the proteins of maize. Science, *37:* 185.

OSBORNE, T. B., AND L. B. MENDEL 1914 Nutritive properties of proteins of the maize kernel. J. Biol. Chem., *18:* 1.

SHOWALTER, M. F., AND R. H. CARR 1922 Characteristic proteins in high- and low-protein corn. J. Am. Chem. Soc., *44:* 2019.

STOKES, J. L., M. GUNNESS, I. M. DWYER AND M. C. CASWELL 1945 Microbiological methods for the determination of amino acids. II. A uniform assay for the ten essential amino acids. J. Biol. Chem., *160:* 35.

STUBBLEFIELD, F. M., AND E. E. DETURK 1940 The composition of corn, oats, and wheat as influenced by soil, soil treatment, seasonal conditions and growth. Soil Sci. Soc. Am. Proc., *5:* 120.

STUDENT 1925 New tables for testing the significance of observations. Metron, *5:* 105.

WOODWORTH, C. M., E. R. LENG AND R. W. JUGENHEIMER 1952 Fifty generations of selection for protein and oil in corn. Agron. J., *44:* 60.

THE INFLUENCE OF VITAMIN B_{12} AND ANTIBIOTICS ON PROTEIN AND ENERGY UTILIZATION IN A LOW PROTEIN DIET [1, 2]

LEON K. KNOEBEL AND ALEX BLACK

Department of Animal Nutrition, The Pennsylvania State College, State College

(Received for publication July 1, 1952)

It is well known that vitamin B_{12} is associated with growth in many species of animals, and it is likewise recognized that the addition of antibiotics to the rations of some of these animals, such as chickens, turkeys, and swine, produces a further growth response over that obtained with vitamin B_{12} alone in a basal ration which is otherwise complete except for these supplements. Since a stimulation of growth is involved in both of these circumstances, there is suggested the possibility that vitamin B_{12} and antibiotics are involved in protein metabolism.

In experiments designed to study this question, Hartman, Dryden and Cary ('49) observed that a deficiency of vitamin B_{12} may have a very deleterious effect upon the growth of rats. From this work they concluded that vitamin B_{12} must play a fundamental role in affecting the capacity of normal mammals to utilize protein. Hove and Hardin ('51) found that rats which were consuming a 10% protein diet gained more weight per gram of protein ingested when vitamin B_{12} was included in the diet than when it was omitted, which

[1] Authorized for publication on June 26, 1952, as paper 1741 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

[2] We are indebted to Chas. Pfizer and Co., Brooklyn, N. Y., for the vitamin B_{12} supplement, Bi-Con 3+, and streptomycin sulfate and to Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., for the aureomycin hydrochloride.

indicated to them that efficiency of protein utilization was influenced by vitamin B_{12}. Further, it was shown by Charkey, Wilgus, Patton and Gassner ('50) that vitamin B_{12} has a possible function in utilization of circulatory amino acids, since a higher level of amino acids was found in the blood stream of B_{12}-deficient chicks than in B_{12}-supplemented birds.

Henry and Kon ('51) found the biological value of casein determined with rats to be higher when supplemented with vitamin B_{12} than when vitamin B_{12} was missing, but true digestibility was unaffected by the vitamin. Although their results demonstrate that the assimilation of nitrogen was less in the absence of vitamin B_{12} than with it, the authors were of the opinion that this relationship was not necessarily specific.

Chow and Barrows ('50) concluded from work with rats that carbohydrate or fat metabolism was more affected by vitamin B_{12} than was protein metabolism, since they found vitamin B_{12} to be without influence on nitrogen retention.

In recent work from this laboratory Black and Bratzler ('52) studied the effect of vitamin B_{12} and antibiotics, singly and in combination, on protein and energy metabolism in rats, and found that protein utilization, as such, was not differentially affected by these supplements when the level of protein in the diet was 18%. In these experiments significantly greater body gains were observed with rats when vitamin B_{12} was added to a diet of low B_{12} content than when it was omitted, but indications were lacking that protein, specifically, was more efficiently utilized than was fat or total gain of energy.

The present experiment was designed to study this problem further by investigating the combined effects of vitamin B_{12} and certain antibiotics on protein and energy utilization in the rat when a low protein diet was fed.

EXPERIMENTAL

This experiment, like the previous one (Black and Bratzler, '52), involved a 70-day growth and metabolism investigation

with albino rats, employing the body balance techniques described by Swift et al. ('34).

The experimental subjects consisted of two groups of 12 litter-mate pairs of albino rats, each animal of a pair being of the same sex and approximately the same weight at the start of the experiment. The distribution of animals according to sex was nearly equal; 7 pairs of males and 5 pairs of females were used. The diets (table 1) for the two

TABLE 1

Composition of diets

	UNSUPPLEMENTED	SUPPLEMENTED
	%	%
Soybean oil meal (solvent)	16.78	16.78
Linseed oil meal (solvent)	6.84	6.84
Salt mixture IV [1]	4.00	4.00
Mazola	5.00	5.00
Dextrin	33.69	33.69
Cerelose	33.69	33.69
Vitamin B_{12} supplement [2]		0.50
Aureomycin hydrochloride		0.0047
Streptomycin sulfate		0.0047

[1] Lichstein et al. ('46).

[2] The vitamin B_{12} supplement was Bi-con 3+, a product of Chas. Pfizer and Co.

Vitamins were added in the following proportions on a kilogram basis: Oleum percomorphum, 570 mg; tocopherols (mixed) 150 mg; choline, 4,000 mg; inositol, 2.0 gm; folic acid, 0.25 μg; biotin, 0.10 μg; thiamine, 5 mg; riboflavin, 5 mg; pyridoxine hydrochloride, 6.25 mg; niacin, 6.25 mg; calcium pantothenate, 50 mg; para-aminobenzoic acid, 95 mg; 2-methyl 1,4-naphthoquinone, 2.5 mg.

groups of animals were identical, except that to one diet there was added a vitamin B_{12} supplement in a quantity sufficient to supply 33 μg of vitamin B_{12} activity per kilogram of diet and 5.5 mg of antibiotic in the form of streptomycin and terramycin per kilogram. Streptomycin sulfate and aureomycin hydrochloride were also added to the supplemented diet in equal quantities so that there were 100 mg of total antibiotic per kilogram of diet.

The rats were fed in accordance with the paired-feeding technique, which involved limiting the amount of feed of

one animal to the amount consumed by the member of the pair eating the least.

In order to compare the feed intake and growth obtained with the paired-feeding technique with those obtained when the feeding was on an ad libitum basis, another group of 8 rats were fed the supplemented diet ad libitum for a 5-week period. Observations in this experiment were limited to only feed intake and growth.

RESULTS AND DISCUSSION

It was evident in the paired-feeding experiment that the rats receiving the unsupplemented diet determined the feed consumption, since of a total of 840 feed offerings there were 436 refusals of feed in the unsupplemented group and only 69 refusals in the supplemented group.

The average amounts of feed eaten, the gains in body nitrogen and fat, and the live weight are given in table 2. Some of these data for the pair-fed group are given both for a 5-week and a 10-week period so that a comparison can be made with the ad libitum-fed animals that were on experiment only 5 weeks.

The pair-fed rats that received the basal diet gained an average of 122 gm in weight, while those on the supplemented diet gained 127 gm in weight. The statistical significance of such a difference, when computed by Student's method as modified by Love ('24), shows odds of 83 to 1 that the difference did not occur by chance alone. Most of the difference in gain in body weight between the two groups was accounted for by gain of fat — the greater amount being in favor of the animals that received the supplemented diet. Odds indicating the significance of this difference were 51 to 1. Thus there was an indication that not only did the supplemented animals gain more weight, but that this gain was of higher energy content. A further observation regarding body composition was the water content; however, these values are not shown in table 2. For the rats on the basal diet the bodies contained 63.7% water and on the supplemented

TABLE 2

Average amounts of feed eaten and gains in live weight, nitrogen and fat

DIET	TIME ON EXP.	METHOD OF FEEDING	FEED EATEN	LIVE WEIGHT		GAIN IN LIVE WEIGHT	NITROGEN OF BODY GAIN	FAT GAINED
				Initial	Final			
	weeks		*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Unsupplemented	5	Paired	292 ± 7 [1]	47 ± 1.1	110 ± 2.3	63 ± 2.1		
Supplemented	5	Paired	292 ± 7	47 ± 1.0	115 ± 2.6	68 ± 2.4		
Unsupplemented	10	Paired	672 ± 14	47 ± 1.1	169 ± 3.9	122 ± 3.8	3.9 ± 0.1	14.6 ± 0.8
Supplemented	10	Paired	672 ± 14	47 ± 1.0	174 ± 4.4	127 ± 4.2	3.9 ± 0.1	18.2 ± 1.5
Unsupplemented	5	Ad libitum	300 ± 8	47 ± 1.1	118 ± 4.0	71 ± 3.9		

[1] Standard error of the mean.

diet this value was 62.5%. In the ad libitum-fed rats the live weight was 118 gm at the end of 5 weeks, compared to 110 gm and 115 gm for the pair-fed rats on the control and supplemented diets, respectively, for the same length of time. It was expected that the ad libitum-fed rats would consume more feed than the pair-fed rats, since feed consumption was limited largely in the pair-fed rats by those on the control ration, but at the end of the 5-week experimental period, differences in feed consumption and weight gains between the two supplemented groups fed by the different feeding techniques were very small (table 2). Obviously the amount of feed consumed by the unsupplemented group in the pair-fed experiment was extremely close to the amount which would be consumed by the supplemented group as a maximum. A slight variability may have been due to the use of different animals, but the magnitude of the difference is sufficiently small to appear unimportant.

Black and Bratzler ('52), using the same type of diet and supplement employed here, but with an 18% protein level, obtained quite significant weight differences in rats on unsupplemented and supplemented diets. The small differences in weight gains obtained in the present work are attributed mainly to the suboptimum level of protein used. This low protein level appears to limit any beneficial effects that the added vitamin B_{12} or antibiotics may produce. It is emphasized that considerably less feed is consumed on such low-protein diets, resulting in a disproportionately smaller amount of feed available for body gain.

The average distribution of feed nitrogen as affected by the vitamin B_{12} and antibiotic supplementation is given in table 3.

Body gains of nitrogen determined either by difference, as in table 3, or by direct analysis as in table 2, were practically identical in both groups, so at the level of protein used neither vitamin B_{12} nor antibiotics functioned by increasing protein utilization.

In other experiments where the diet contained 18% protein and feeding was on an ad libitum basis, Black and Bratzler ('52) found a much greater assimilation of nitrogen when vitamin B_{12} was present in the ration than when it was deficient; however, they did not find this relationship to be specific for protein. Gains of fat and total gain of energy were similarly affected. They also found that when the feed intake was on a pair-feeding basis and the protein intake still at the 18% level, neither assimilation nor utilization of nitrogen was affected by vitamin B_{12}.

TABLE 3

Distribution of feed nitrogen as affected by vitamin B_{12} and antibiotic supplementation

DIET	UNSUPPLEMENTED	SUPPLEMENTED
Nitrogen		
Food, gm	10.96 ± 0.2 [1]	10.96 ± 0.2
Feces, gm	2.11 ± 0.0	1.99 ± 0.0
Digested, gm	8.85	8.97
% of food N	80.8 ± 0.3	81.9 ± 0.5
Urine, gm	4.54	4.85
% of food N	41.4 ± 0.7	44.4 ± 0.7
Gain of N, gm	3.88 ± 0.1	3.91 ± 0.1

[1] Standard error of the mean.

A comparison of the data of the present experiment with those of Charkey et al. ('50) cannot be made on a direct basis, since the level of protein in the diets was very different and excretion of nitrogen was not determined by Charkey and co-workers. It is interesting to note, however, that Charkey postulated that there was less renal loss in their experiment in the vitamin B_{12}-supplemented chicks than in those without this supplement in their diet. In the present experiment renal loss was measured and was greater in the rats receiving the vitamin B_{12} and antibiotics than in the controls. Charkey et al. ('50) were of the opinion that the lowered amino acid content of the blood and the greater body gain of the vitamin B_{12}-supplemented chicks were associated with a lowered renal wastage.

The method used by Hove and Hardin ('51) in calculating protein utilization is not entirely satisfactory, since they assume that all gain in weight is of equal composition. Some or all of the increased weight gains could have consisted of fat or water. While the present work is in complete agreement with that of Hove and Hardin and that of Hartman, Dryden and Cary ('49) as far as the influence of vitamin B_{12} on growth is concerned, it lacks specific indication that vitamin B_{12} has a greater influence on protein metabolism than on fat metabolism or simply on total gain of energy. In this

TABLE 4

Distribution of total feed energy as affected by vitamin B_{12} and antibiotic supplementation

DIET	UNSUPPLEMENTED	SUPPLEMENTED
Energy value in Cal.		
Food	2,623 ± 55 [1]	2,623 ± 55
Feces	221 ± 5	265 ± 8
Digested	2,401 ± 50	2,358 ± 49
Urine	55 ± 2	46 ± 1
Metabolized	2,347 ± 49	2,312 ± 48
Body gain: total	284 ± 12	318 ± 14
as protein	148 ± 5	133 ± 7
as fat	136 ± 8	185 ± 12
Heat production	2,063 ± 41	1,994 ± 41

[1] Standard error of the mean.

respect the conclusion of Henry and Kon ('51) that vitamin B_{12} lacks specificity in regard to protein utilization appears to be the most logical, but further experimentation of a somewhat different nature than that so far reported will be required to show clearly the role of vitamin B_{12} in the building of body tissue.

From the distribution of total feed energy as given in table 4 it is shown that the energy of the feces of the unsupplemented animals was less than that of the feces of the supplemented animals, resulting in a higher digestible energy for the control group, this being highly significant, as is shown by odds of more than 10,000 to 1. The magnitude of

this difference in digestible energy is reflected almost directly in the metabolizable energy, as the urinary energy is very similar in both groups. Consequently, the control rats had a significantly higher amount of metabolizable energy available, as shown by odds of 10,000 to 1, but in spite of the greater metabolizable energy available, the body gain of energy was less in this group. In the unsupplemented animals 10.8% of the feed energy went into body gain as compared to 12.2% for the supplemented animals, with most of the difference in body gain of energy being represented by the larger gain of energy as fat. The significance of the difference in energy gained as fat is shown by odds of 494 to 1. This observation on gain of fat is in confirmation of earlier work in which a similar method of feeding was employed (Black and Bratzler, '52).

The final difference observed in the partition of energy was with respect to heat production. The unsupplemented animals used 78.7% of the feed energy for heat production, which was significantly higher than the 76.0% of the feed energy used by the supplemented animals for the same purpose (odds 10,000 to 1).

Since heat production is a waste of feed energy insofar as growth is concerned, the vitamin B_{12} and antibiotic supplements evidently were somewhat more effective in conserving some of this energy for body gain than was the unsupplemented diet. It is interesting to note that even though metabolizable energy was greater in the unsupplemented rats, body gain of energy was higher in the supplemented animals. This can mean that once the feed energy is absorbed from the intestinal tract the supplements play a role in the utilization of this energy for body gain.

In line with this thought is the postulation that antibiotics have an effect on the intestinal flora of the rat by favoring the production of those bacteria which can synthesize vitamin B_{12}, and, at the same time, suppressing the growth of certain microorganisms that utilize vitamin B_{12} for their

growth and thus make it unavailable to the rat (Cravioto-Monoz et al., '51).

Much work has been done showing that vitamin B_{12} and various antibiotics influence growth in many animals, but the exact mechanism by which this is accomplished is still unknown. It is thought, however, that increased feed consumption induced by the supplements is one of the major factors involved in this process. Also, in this work it has been shown that food is better utilized after absorption from the intestinal tract when vitamin B_{12} and antibiotics are present than when these supplements are absent.

SUMMARY

The effects of a combination of vitamin B_{12} and antibiotics on protein and energy utilization were investigated in a 70-day growth and body balance experiment with 12 litter-mate pairs of growing albino rats. One animal of each pair was fed an unsupplemented diet compounded to contain adequate quantities of all known essential nutrients except vitamin B_{12}, while its litter mate was fed the same diet, in equivalent amounts (paired-feeding), plus a supplement containing vitamin B_{12} and antibiotics. The protein in the diet was of vegetable origin and was fed at a 10% level.

The rats on the supplemented diet gained somewhat more live weight, stored more fat and less water, had less digestible and metabolizable energy and produced less heat than the animals on the unsupplemented diet.

At the level of protein fed in this experiment there was no indication that a specific relationship existed between the utilization of nitrogen and the supplements used, which is in confirmation of previous work in which a higher level of protein was fed. The utilization of energy-producing nutriment absorbed from the intestinal tract was shown to be improved by the presence of vitamin B_{12} and antibiotics in the diet.

LITERATURE CITED

BLACK, ALEX, AND J. W. BRATZLER 1952 The effects of an APF supplement, vitamin B_{12}, and streptomycin on the metabolism of the rat. J. Nutrition, *47:* 1.

CHARKEY, L. W., H. S. WILGUS, A. R. PATTON AND F. X. GASSNER 1950 Vitamin B_{12} in amino acid metabolism. Proc. Soc. Exp. Biol. Med., *73:* 21.

CHOW, B. F., AND L. BARROWS 1950 Role of vitamin B_{12} on nitrogen retention of rats fed on soybean protein diets at different caloric levels. Fed. Proc., *9:* 354.

CRAVIOTO-MONOZ, J., H. G. PONCHER AND H. A. WAISMAN 1951 Vitamin B_{12} sparing action of aureomycin in the rat. Proc. Soc. Exp. Biol. Med., *77:* 18.

HARTMAN, A. M., L. P. DRYDEN AND C. A. CARY 1949 A role of vitamin B_{12} in the normal mammal. Arch. Biochem., *23:* 165.

HENRY, K. M., AND S. K. KON 1951 Vitamin B_{12} and the biological value of proteins. Biochem. J., *48:* xi.

HOVE, E. L., AND J. H. HARDIN 1951 Effect of vitamins E, B_{12}, and folacin on CCl_4 toxicity and use of protein in rats. Proc. Soc. Exp. Biol. Med., *77:* 502.

LICHSTEIN, H. C., K. B. MCCALL, EDNA B. KEARNEY, C. A. ELVEHJEM AND P. F. CLARK 1946 Effect of minerals on susceptibility of Swiss mice to Theiler's virus. Ibid., *62:* 279.

LOVE, H. H. 1924 A modification of Student's tables for use in interpreting experimental results. J. Am. Soc. Agron., *16:* 68.

SWIFT, R. W., O. J. KAHLENBERG, L. VORIS AND E. B. FORBES 1934 The utilization of energy-producing nutriment and protein as affected by individual nutrient deficiencies. I. The effects of cystine deficiency. J. Nutrition, *8:* 197.

CHRONIC SODIUM CHLORIDE TOXICITY IN THE ALBINO RAT

I. GROWTH ON A PURIFIED DIET CONTAINING VARIOUS LEVELS OF SODIUM CHLORIDE

GEORGE R. MENEELY, ROBERT G. TUCKER AND WILLIAM J. DARBY

Radioisotope Unit and Research Laboratory, Thayer Veterans Administration Hospital, and the Departments of Medicine and Biochemistry, Vanderbilt University School of Medicine
Nashville, Tennessee

(Received for publication July 25, 1952)

The importance of dietary salt has been recognized from time immemorial, and dietary sodium has been suspected for over 30 years as a primary cause of arterial hypertension in man (Allen and Sherrill, '22); yet little evidence has accumulated on the chronic toxicity of sodium chloride. The more primitive type of renal glomerulus in fowl as compared to the mammal makes it more susceptible to sodium chloride poisoning; hence most studies have been made on birds (French, '35; Krakower and Goettsch, '45; Lenel, Katz and Rodbard, '48). Campbell ('46) reported on the growth and reproduction of the rat on diets containing 1.32, 2.59 and 5.06% of sodium chloride. No great differences were observed in growth, apparent well-being or ability to reproduce. There were some indications of renal damage at the highest level of sodium chloride intake. Meyer et al. ('50) found that high level feeding of sodium retarded somewhat the growth of rats.

This report covers the growth, ration consumption, early mortality, and general observations on rats fed a purified diet containing sodium chloride at 7 different levels.

EXPERIMENTAL

Ration I contained casein, vitamin-test,[1] 25.0%; cane sugar, 51.9%; shortening, all-vegetable (Crisco), 20.0%; a mineral mixture,[2] 2.9%; and a vitamin mixture,[3] 0.2%. It is a low-sodium diet (no sodium chloride added but containing 0.01% of sodium chloride by analysis). Rations II to VII, inclusive, were made by intimately mixing ration I with the calculated amount of sodium chloride, C. P. powder, in a Hobart mixer. Ration II contained an adequate reasonable amount of sodium chloride equivalent to incorporating the Hubbell, Mendel and Wakeman ('37) salt mixture at about a 2.0% level. Rations III to VII, inclusive, were progressively higher in sodium chloride content, 20, 40, 50, 60 and 70 times the Hubbell, Mendel and Wakeman formula fed at a 2% level. Rations were made fresh at no more than 15-day intervals and were stored at 7°C. until placed in feed cups.

Male, Sprague-Dawley, albino rats, 33 to 40 days old and weighing from 86 to 154 gm, were divided into 7 matched groups and placed on demineralized water and the purified diets ad libitum. The rats were kept in steel wire cages (several rats per cage) in a constant temperature room (27° ± 2°C.). They were fed, watered and observed daily, and individually weighed and carefully examined weekly. The rate of food and water consumption was studied for the first 5 weeks of the experimental regimen. Scatter-proof feed

[1] Manufactured by Nutritional Biochemicals, Inc., Cleveland, Ohio.

[2] The mineral mixture is a modification of the Hubbell, Mendel and Wakeman ('37) formula by weight, as follows: $CaCO_3$, A. R., 543.0; KH_2PO_4, A. R., 212.0; KCl, A. R., 112.0; $FePO_4$, N. F., 38.1; $MgCO_3$, A. R., 25.0; $MgSO_4$, A. R., 16.0; $KF \cdot 2H_2O$, A. R., 2.20; $CuSO_4 \cdot 5H_2O$. A. R., 1.41; $ZnSO_4 \cdot 7H_2O$, U. S. P., 0.90; $MnSO_4 \cdot H_2O$, A. R., 0.39; $AlK(SO_4)_2 \cdot 12H_2O$, U.S.P., 0.31; KI, C. P., 0.08; and $CoSO_4 \cdot 7H_2O$, C. P., 0.01.

[3] The vitamin supplementation in weight per kilogram of ration I was as follows: thiamine hydrochloride, 5.0 mg; riboflavin, 5.0 mg; pyridoxine hydrochloride, 2.5 mg; vitamin B_{12}, 40.0 μg; *i*-inositol, 1.0 gm; *p*-aminobenzoic acid, 2.5 mg; biotin 24.0 μg; niacin, 20.0 mg; calcium pantothenate, 30.0 mg; choline chloride, 1.0 gm; menadione bisulfite, 4.8 mg; α-tocopherol, 30.0 mg; folic acid, 1.0 mg; and concentrated oleovitamin A and D, U.S.P., 0.4 gm.

cups [4] and inverted wide mouth bottles with stainless steel water feeder tubes [5] were used.

RESULTS

Growth

Table 1 lists the per cent of sodium chloride in the ration and the group means and standard deviations of the gross body weight fortnightly for the first 20 weeks of the experimental regimen. This tabulation includes only those rats which exhibited good health throughout the test period. Excluded here were some 11% of the rats which developed abnormalities such as intercurrent infection or which sustained injuries and were removed from the colony. This omission was made in order to keep the number of animals in each group constant throughout the 20-week period. However, the growth pattern of the excluded animals did not differ from that of their respective group until edema or other diseased states intervened. A separate report is to be made on the pathologic changes observed in these animals (Meneely et al., '52).

Food consumption

Table 2 lists the rate of food consumption during the first 5 weeks of the experimental regimen. The values are the weekly means expressed as grams of food per rat per day. These values represent an over-estimation of the amount of food eaten by the rats, because some undetermined wastage occurred. The semi-plastic consistency of the food and the scatter-proof feed cups tended to reduce the food wastage to a minimum.

Water consumption

Table 3 lists the rate of water consumption during the first 5 weeks of the experimental regimen. The values are the

[4] Manufactured by C. H. Sommers and Associates, Cincinnati, Ohio.
[5] See footnote 4.

TABLE 1

Growth data (gross body weight in grams at bi-weekly intervals)

TIME IN WEEKS	RATION I 0.01% NaCl		RATION II 0.15% NaCl		RATION III 2.8% NaCl		RATION IV 5.6% NaCl		RATION V 7.0% NaCl		RATION VI 8.4% NaCl		RATION VII 9.8% NaCl	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Initial	116	19	119	17	118	17	117	19	121	17	118	21	121	22
2	170	30	184	26	179	18	176	21	180	21	176	20	173	21
4	207	41	251	28	244	24	229	19	244	22	237	22	228	21
6	226	45	296	29	297	28	276	22	290	28	277	27	269	23
8	256	44	334	31	330	28	315	24	322	30	313	29	301	27
10	281	49	360	32	354	30	339	24	341	31	334	31	324	25
12	315	58	392	34	380	29	365	23	365	32	353	29	341	25
14	340	55	412	36	393	30	374	24	377	33	367	30	349	24
16	359	60	432	38	412	33	394	24	396	35	385	36	368	28
18	388	56	454	38	435	34	414	27	411	35	399	37	383	26
20	416	43	472	39	449	34	432	26	424	36	413	35	395	28
N	10		30		29		28		24		26		25	

weekly means expressed as milliliters of water per rat per day. These values also represent some over-estimation of the water drunk by the rats as an unavoidable amount of spillage occurred.

TABLE 2

Mean ration consumption. All figures are in grams per rat per day

RATION	I	II	III	IV	V	VI	VII
% NaCl	0.01	0.15	2.8	5.6	7.0	8.4	9.8
1st Week	11.1	11.4	11.4	11.5	12.6	12.3	11.7
2nd Week	10.3	12.4	12.8	12.9	14.0	13.7	13.3
3rd Week	10.5	13.4	13.3	13.5	13.8	14.3	13.9
4th Week	10.4	14.2	13.7	12.7	14.7	15.6	15.0
5th Week	10.2	13.9	14.0	14.8	14.6	14.6	13.9
Mean	10.5	13.1	13.0	13.1	13.9	14.1	13.6

TABLE 3

Mean water consumption. All figures are in milliliters per rat per day

RATION	I	II	III	IV	V	VI	VII
% NaCl	0.01	0.15	2.8	5.6	7.0	8.4	9.8
1st Week	24.0	26.4	47.9	55.7	60.0	72.1	73.4
2nd Week	28.6	30.2	40.6	63.4	71.9	77.8	84.9
3rd Week	29.4	38.9	46.1	65.8	76.1	84.0	90.0
4th Week	30.8	45.2	58.0	65.1	82.9	102.7	91.2
5th Week	25.0	49.0	63.1	74.3	87.7	104.1	89.2
Mean	27.6	37.9	51.1	64.9	75.7	88.1	85.7

Mortality and morbidity

The rats grew well on all of the experimental rations. Only 17 rats out of a total of 193 died or were sacrificed during the 20-week period covered in this report. Table 4 summarizes the mortality data. The morbidity included two rats on ration IV with mechanical injury to the snout, one rat on ration V which contracted respiratory infection and one rat on ration VI which developed edema.

DISCUSSION

The diet herein described is of known composition, is reproducible from available, chemically pure nutrients, and is of a desirable consistency for feeding to small animals. It is very low in cholesterol, the only source being the concentrated oleovitamin A and D oil. The diet contains adequate amounts of all vitamins which are known to be required by the rat. In addition a few vitamins are incorporated of which, presumably, the rat does not require a dietary source, the reason being that sufficiently long-term studies have not been

TABLE 4

Mortality experienced during the first 20 weeks on the purified rations

RATION	CAUSE OF DEATH	NUMBER
I	Respiratory infection	1
II	Fractured nose	1
III	Respiratory infection	1
IV		0
V	Renal failure with edema	2
	Respiratory infection	2
	Fractured nose	1
VI	Renal failure with edema	2
	Respiratory infection	1
VII	Renal failure with edema	4
	Renal failure without edema	1
	Accidental hemorrhage	1

reported in every case, and it was deemed desirable not to risk the occurrence of vitamin deficiencies in the present long-term study.

The sodium chloride added to the rations was in a powdered form, freshly screened to render it uniform, and adequately mixed into the diet at high speed in a Hobart mixer. The rat had no chance to reject the added salt from his diet. The dispersion was such that if the rat ate any of the ration, he was forced to ingest the calculated fraction of sodium chloride.

The low-sodium diet (ration I) caused mild anorexia. The small group of rats on this diet ate less, drank less water,

and grew more slowly than did the controls (ration II). These animals exhibited a continuous salt hunger. This was manifest by their habit of licking any object in the cage, particularly wherever fingerprints were left. There was considerable variation in the rate of growth of the animals on ration I as demonstrated by the large standard deviations in gross body weight (table 1).

The high sodium chloride diets (rations III to VII, inclusive) depressed the rate of growth. Table 5 indicates the significance of the differences between the means of the control animals and the other 6 groups. Although the data on

TABLE 5

The probability (P) that the mean body weight of the sample differs from the mean body weight of the controls (ration II). Values in italics indicate that the difference is statistically significant

RATION	START	4 WEEKS	8 WEEKS	12 WEEKS	16 WEEKS	20 WEEKS
I	$>.6$	*$<.01$*	*$<.01$*	*$<.01$*	*$<.01$*	*$<.01$*
III	$>.6$	$>.3$	$>.4$	$>.1$	*$<.05$*	*$<.02$*
IV	$>.5$	*$<.01$*	*$<.01$*	*$<.01$*	*$<.01$*	*$<.01$*
V	$>.8$	$>.3$	$>.1$	*$<.01$*	*$<.02$*	*$<.01$*
VI	$>.8$	*$<.05$*	*$<.01$*	*$<.01$*	*$<.01$*	*$<.01$*
VII	$>.6$	*$<.01$*	*$<.01$*	*$<.01$*	*$<.01$*	*$<.01$*

ration consumption indicate that the rats on high salt diets ate as much or more than the controls, they did not consume as many calories as did the control animals and, consequently, did not gain as rapidly in gross body weight. The fact that most of the rats on the high salt diets grew and thrived demonstrates the remarkable ability of the rat kidney to excrete enormous quantities of salt. As anticipated, these rats exhibited a marked polydipsia and polyuria.

Although food and water spillage by the rat introduces some error into the data on ration consumption (tables 2 and 3), the relative values are reasonably correct. Trends are apparent from a casual inspection of the data. There can be little doubt that the low salt regimen had a depressing

effect on the appetite and that the high salt diets caused thirst but did not depress the appetite to a marked degree.

The general appearance of the rats on the 7 purified diets was good throughout the test period with the following exception. When first placed on the high salt diets, many of the animals developed a condition of extreme dehydration characterized by marked porphyrin staining of the hair and extremities. This occurred during the first week of the experiment. By the end of the second week the condition had almost disappeared and did not recur. The rats had successfully adapted to the high-sodium diet.

Of the 17 deaths occurring during the 20-week period (table 4) only 9 were directly attributable to dietary stress. Less than 5% of the total number of animals involved succumbed to pathologic changes induced by sodium chloride toxicity or 10% of the animals on the three higher levels of sodium intake.

Sporadic renal damage was manifest by the sudden appearance of massive edema. The extracellular fluid measured with radioactive sodium-24 rose from control values of $26.2 \pm 1.88\%$ of body weight to 57.3 and 57.8% of body weight in two rats. During the week of most rapid edema formation, body weight gains averaged 62.8 gm for 9 edematous rats in contrast to an average gain of 10.1 gm a fortnight earlier for the same animals. The edematous rats were characterized by marked anemia, hypoproteinemia, lipemia, and azotemia. Histologic examination of the kidneys revealed extensive glomerular and tubular lesions. The pathology of these rats will be reported when the study is completed.

The surviving animals on all 7 rations were of uniform, healthy appearance indistinguishable with respect to fur, skin, color and behavior when handled from controls fed on Purina Chow. The size of the animals was a notable exception.[6]

[6] The control rats on ration II actually exceeded the Purina Chow-fed rats in rate of growth. However, the test was not legitimate, because the Chow-fed rats were the extremes in weight rejected from the sample at the start of the experiment, and the data are omitted specifically for that reason.

The rate of growth on ration II (controls) compares favorably with other reports in the literature (Copping et al., '51; Freudenberger, '32; Mendel and Hubbell, '35; Zucker et al., '41). However, the "rapidly growing" rats of Pickens et al. ('40), and the small group of rats raised on a purified diet by Mayer ('48) grew more rapidly than did our control animals.

SUMMARY

Young male rats have been raised on a purified diet, containing 7 different levels of sodium chloride, and water ad libitum with the following results:

Rats fed diets high (2.8 to 9.8%) and low (0.01%) in sodium chloride grew more slowly than did rats on a control ration (0.15% NaCl).

High sodium chloride feeding caused polydipsia and polyuria but did not have a marked effect on appetite. A low-sodium diet caused mild anorexia.

Edema developed during this first 20-week period in 10% of the rats fed diets containing from 7.0 to 9.8% of sodium chloride. The majority of the animals (89%) remained in excellent health throughout the 20 weeks.

LITERATURE CITED

ALLEN, F. M., AND J. W. SHERRILL 1922 The treatment of arterial hypertension. J. Metabolic Research, *2:* 429.

CAMPBELL, H. L. 1946 Sodium chloride as an adjunct to a diet of whole wheat and whole milk. Am. J. Physiol., *147:* 340.

COPPING, A. M., P. J. CROWE AND V. R. G. POND 1951 The growth response of rats to purified diets. Brit. J. Nutrition, *5:* 68.

FRENCH, M. H. 1935 Salt poisoning of fowls. Ann. Rept. Dept. Vet. Sci. Animal Husbandry, Tanganyika Terr., p. 25. Chem. Abstr., *31:* 3531.

FREUDENBERGER, C. B. 1932 A comparison of the Wistar albino and the Long-Evans hybrid strain of the Norway rat. Am. J. Anat., *50:* 293.

HUBBELL, R. B., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. J. Nutrition, *14:* 273.

KRAKOWER, C. A., AND M. GOETTSCH 1945 Effect of excessive ingestion of sodium chloride on the chick, with particular reference to renal changes. Arch. Path., *40:* 209.

LENEL, R., L. N. KATZ AND S. RODBARD 1948 Arterial hypertension in the chicken. Am. J. Physiol., *152:* 557.

MAYER, J. 1948 Growth characteristics of rats fed a synthetic diet. Growth, *12:* 341.

MENDEL, L. B., AND R. B. HUBBELL 1935 The relation of the rate of growth to diet. III. A comparison of stock rations used in the breeding colony at the Connecticut Agricultural Experiment Station. J. Nutrition, *10:* 557.

MENEELY, G. R., R. G. TUCKER, S. H. AUERBACH AND W. J. DARBY 1952 Renal damage in rats fed large quantities of sodium chloride. J. Clin. Invest., *31:* 650.

MEYER, J. H., R. R. GRUNERT, M. T. ZEPPLIN, R. H. GRUMMER, G. BOHSTEDT AND P. H. PHILLIPS 1950 Effect of dietary levels of sodium and potassium on growth and on concentrations in blood plasma and tissues of white rat. Am. J. Physiol., *162:* 182.

PICKENS, M., W. E. ANDERSON AND A. H. SMITH 1940 The composition of gains made by rats on diets promoting different rates of gain. J. Nutrition, *20:* 351.

ZUCKER, T. F., L. HALL, M. YOUNG AND L. ZUCKER 1941 The growth curve of the albino rat in relation to diet. Ibid., *22:* 123.

THE METHIONINE REQUIREMENT FOR THE GROWTH OF SWINE

L. V. CURTIN,[1] J. K. LOOSLI, JEAN ABRAHAM,
H. H. WILLIAMS AND L. A. MAYNARD
Departments of Animal Husbandry and Biochemistry and Nutrition, Cornell University, Ithaca, New York

(Received for publication July 31, 1952)

The report of Bell et al. ('50) showed that methionine is an essential amino acid for the growth of swine. The authors suggested that on a diet containing 10% protein, the methionine requirement for swine is between 0.07 and 0.27% of the ration. Later Shelton et al. ('51) reported excellent growth of pigs on a 21% protein ration containing oxidized casein and gelatin plus tryptophan, and supplemented to contain 0.6% methionine. They demonstrated further that 0.3% of L-cystine could replace one-half of the methionine. More recently Curtin et al. ('52) showed that when weanling pigs were fed a soybean oil meal-purified ration containing 22% protein the methionine requirement did not exceed 0.37% in the presence of 0.38% cystine.

In the present work, a methionine deficient ration containing isolated soybean protein [2] and brewers' dried yeast was fed to weanling pigs in two experiments to obtain additional data on the methionine requirement of swine. The studies of Grau and Almquist ('43) demonstrated that isolated soybean protein is deficient in the sulfur containing amino acids for optimum chick growth. Likewise, brewers' dried yeast is low in methionine (Block and Mitchell, '46–'47).

[1] Present address: Technical Division, The Buckeye Cotton Oil Co., Cincinnati, Ohio.

[2] "Alpha protein" produced commercially by the Glidden Co., Chicago, Ill.

PROCEDURE

The basal rations fed are shown in table 1. No choline chloride was added to the ration since it has been shown that choline has a sparing effect on the methionine requirement for swine (Dyer et al., '49). Ration A was fed in the first trial

TABLE 1

Composition of basal rations

CONSTITUENTS	A	B
Isolated soybean protein [1]	21.3	
Isolated soybean protein, washed [2]		20.6
Brewers' dried yeast	5.0	5.0
Glucose (cerelose)	25.0	25.0
Lard	3.9	
Corn oil		4.0
Ruffex [3]	3.9	4.0
Minerals [4]	4.0	5.0
Vitamins and antibiotic [5]	+	+
Starch [6]	36.9	36.4

[1] "Alpha protein" produced commercially by the Glidden Co., Chicago, Illinois.

[2] "Alpha protein" that had been washed with water as described in the text.

[3] A commercial rice hull preparation by the Fisher Scientific Co.

[4] The following ingredients were included in the mineral mixture for rations A and B, respectively: $CaHPO_4 \cdot 2H_2O$, 49.6% and 70.0%; $CaCO_3$, 24.8% and 10.0%; NaCl, 24.8% and 20.0%; $FeSO_4 \cdot 7H_2O$, 0.48% and 0.44%; KI, 0.03% and 0.03%; $MnSO_4 \cdot 5H_2O$, 0.06% and 0.08%; $CuSO_4 \cdot 5H_2O$, 0.03% and 0.03%; $CoCl_2$, 0.015% and 0.013%; and $ZnCl_2$, 0.015% and 0.013%.

[5] The following vitamins were added to each 100 lb. of basal mixture: thiamine hydrochloride, 95 mg; riboflavin, 140 mg; niacin, 468 mg; calcium pantothenate, 670 mg; pyridoxine hydrochloride, 110 mg; folic acid, 27 mg; vitamin A, 100,000 I.U. and vitamin D, 10,000 I.U., both supplied by a concentrated fish oil; mixed tocopherols, 9 mg; and vitamin B_{12}, 1 mg. Aureomycin hydrochloride was added at the rate of 500 mg per 100 lb. In addition, each pig in trial A received 3.4 mg of mixed tocopherols orally twice during the experiment.

[6] In the formation of experimental rations, DL-methionine, starch, and glycine were added to bring the total ingredients to 100%.

and differed from ration B in that ration B contained water-washed isolated soybean protein in place of the commercial isolated soybean protein, corn oil in place of lard, and a slightly different mineral mixture. Some difficulties were encountered in other studies subsequent to trial A which were believed to

be caused by sulfite present in the isolated soybean protein. The isolated soybean protein was washed with water by the following procedure. The dry commercial isolated soybean protein was autoclaved in shallow pans for 10 minutes at 10-lb. pressure. The autoclaved protein was then mixed in 30 to 35-lb. batches with approximately 15 gallons of tap water. The protein was allowed to settle, and the supernatant liquid was then siphoned off. Each batch of protein was washed with

TABLE 2

Amino acid content of the basal rations [1]

	PER CENT OF RATION	
	A	B
Arginine	1.52	1.60
Histidine	0.51	0.52
Isoleucine	1.11	1.19
Leucine	1.64	1.81
Lysine	1.34	1.31
Methionine [2]	0.26	0.25
Cystine [3]	0.26	0.27
Phenylalanine	1.15	1.21
Tyrosine	0.70	0.63
Threonine	0.78	0.78
Tryptophan	0.22	0.22
Valine	1.32	1.44

[1] Determined by microbiological techniques to be published in detail later.

[2] Before supplementation with DL-methionine.

[3] Cystine analysis for isolated soybean protein was determined by Dr. R. J. Block, New York Medical College, New York, N. Y.

three changes of water. After the third wash, the protein was pressed as free from water as possible in a screw press and was then dried in shallow trays in a drying room with a maximum temperature of 70°C. The washed, dried, and ground protein had the same physical appearance as the unwashed product. Miller ('51) found that the isolated soybean protein washed in this manner contained not more than 0.1% sulfite calculated as sodium sulfite as compared with approximately 1.1% in the unwashed protein.

The amino acid analyses of the two basal rations, shown in table 2, indicate that there was no significant change in the content of the essential amino acids caused by washing the protein. The only difference noted in the chemical composition of the unwashed and washed soybean protein was the ash content. The ash was reduced from 1.7% to 0.5% by the washing process.

In trial A, two levels of DL-methionine were added to the basal mixture to give three experimental rations of varying methionine content. A 4th lot of pigs was fed the basal ration plus 0.1% choline chloride.

In trial B, three levels of DL-methionine were added to the basal mixture to give 4 experimental rations of varying methionine content. Glycine was added to equalize the protein ($N \times 6.25$) content of each ration at 22%. A 35-day feeding period was used in trial A. The pigs in trial B were fed for only 28 days.

Growth rates, feed efficiency, and nitrogen balances were used as measures of the responses of weanling Yorkshire pigs to the different treatments. Daily feed intakes were equalized weekly at approximately 5% of the body weight. For nitrogen balances, fecal and urine collections were made over a 5-day period following a preliminary period of a minimum of 10 days.

RESULTS

The results of the two tests are given in tables 3 and 4. Since the initial weights of the lots in trial B differed considerably, the average daily gains are also presented as percentages of the initial weights. The data reported in table 3 clearly show that ration A was deficient in methionine. There was a 20% improvement in growth rate ($P \leqq .02$) on 12% less feed ($P \leqq .01$) when the ration was supplemented with 0.3% DL-methionine to make 0.56% total methionine. When the ration contained 0.36% methionine, neither the rate of gain nor the feed efficiency was as good as when the diet contained 0.56% methionine. The data for the apparently digested nitrogen retained in the body show that the increased growth of pigs re-

ceiving supplements of methionine involved storage in the form of protein tissue.

The results of trial B show that the basal ration plus 0.2% DL-methionine, containing a total of 0.45% methionine in the ration, was adequate for maximum growth, feed utilization

TABLE 3

Average data for pigs receiving various levels of DL-*methionine in the isolated soybean protein ration A*

	LOT NO. AND RATION			
	1	2	3	4
	Basal	Basal + 0.1% methionine	Basal + 0.3% methionine	Basal + 0.1% choline chloride
Total methionine in ration (%)	0.26	0.36	0.56	0.26
Total methionine plus cystine in ration (%)	0.52	0.62	0.82	0.52
Number of pigs	4 [1]	6	6	6
Days on test	35	35	35	35
Initial weight (lb.)	35.0	33.6	34.1	33.9
Daily gain (lb.)	1.01	1.07	1.21 [2]	1.03
Daily gain (% of initial weight)	2.88	3.18	3.55	3.04
Daily feed intake (% of body weight)	4.24	4.16	4.22	4.23
Feed per 100 lb. gain (lb.)	221	204 [3]	193 [4]	213
Apparent digestibility of nitrogen (%)	94	94	94	95
Apparently digested nitrogen retained (%)	43	50	52	44

[1] Two pigs were removed because of pneumonia.
[2] Significant over lots one and 4 at 2% level.
Significant over lot two at 5% level.
[3] Significant over lot one at 2% level.
[4] Significant over lots one and 4 at 1% level.
Significant over lot two at 5% level.

and nitrogen retention. The pigs grew 8% faster on 7% less feed when the basal ration was supplemented with 0.2% DL-methionine. The growth rates for the three methionine supplemented lots were similar when expressed as percentages of the initial weights. Also, there were no differences in feed efficiency for the three lots receiving added methionine. Al-

though the differences in rate of gain and feed efficiency between the unsupplemented basal lot and the lot that received the ration containing 0.45% methionine appear real, none of the differences in rate of gain or feed efficiency was statistically significant. The results in trial A indicate that a level of 0.36% methionine in the ration is not adequate for optimum growth; and in trial B, the ration was not improved by the addition of

TABLE 4

Average data for pigs receiving various levels of DL-*methionine in the washed isolated soybean protein ration B*

	LOT NO. AND RATION			
	1 Basal	2 Basal + 0.2% methionine	3 Basal + 0.4% methionine	4 Basal + 0.6% methionine
Total methionine in ration (%)	0.25	0.45	0.65	0.85
Total methionine plus cystine in ration (%)	0.52	0.72	0.92	1.12
Number of pigs	5	3 [1]	5	5
Days on test	28	28	28	28
Initial weight (lb.)	29.1	32.1	29.4	29.8
Daily gain (lb.)	0.77	0.92	0.85	0.84
Daily gain (% of initial weight)	2.65	2.85	2.87	2.82
Daily feed intake (% of body weight)	4.46	4.38	4.41	4.36
Feed per 100 lb. gain (lb.)	231	215	215	216
Apparent digestibility of nitrogen (%)	92	91	93	91
Apparently digested nitrogen retained (%)	36	42	36	39

[1] Two pigs died during the experiment. See text for details.

methionine beyond a level of 0.45% of the ration. From these data it appears that for the 22% protein ration used, the methionine requirement for growth of weanling pigs is approximately 0.45% of the ration, when the ration contains 0.27% cystine, or a total of 0.72% of methionine plus cystine.

In trial A, there was no significant improvement in growth or feed required per unit of gain when the basal ration was supplemented with 0.1% choline chloride. This indicates that

there was no deficiency of choline chloride or labile methyl groups in the basal ration. Analysis of the "alpha protein" and brewers' dried yeast showed that the ration contained 0.024% choline.

The average gains of the pigs in trial A were greater than the expected daily gain for pigs of that weight as given by the National Research Council ('50). The growth rates of pigs in trial B were slower than the growth rates of comparable lots of pigs in trial A. Two reasons are apparent for this difference. The pigs in trial A weighed more at the start of the test and were fed for 35 days in comparison to 28 days in trial B. Since the manner of feeding limited the individual daily feed intake to approximately 5% of the body weight, the larger pigs would be expected to gain at a faster rate on comparable rations. Likewise, with a longer feeding period, the pigs would gain more because of their greater size toward the end of the feeding period.

DISCUSSION

During the second week in trial B, one pig in lot two and one in lot 4 suddenly developed a flaccid type of paralysis. Both pigs recovered within 48 hours following an injection of 333 mg of thiamine hydrochloride.

One pig in lot two of trial B died quite suddenly on the 16th day of the experiment. He had been eating well and making excellent gains (0.91 lb. per day). Just prior to death, he was bloated and appeared to be in great pain. Autopsy showed no cause for death although bloat was evident. On the 25th day, another pig in lot two died quite suddenly. Autopsy disclosed no apparent cause for death although some evidence of bloat was observed. It is interesting to note that Bell et al. ('50) also reported that two pigs receiving a soybean oil meal-purified ration died suddenly. These pigs also showed bloat; however, in addition they were edematous in the inner thigh in the scrotum region and had meningeal hemorrhages. No final diagnosis was made.

Lyman and Elvehjem ('51) reported studies in which as much as 70% of the thiamine was destroyed in a purified ration

in 6 days. When it became evident that the rations in trial B might be deficient in thiamine, new rations were mixed. These rations were kept at a temperature of approximately — 18°C. for the last 10 days of the experiment, and for one pig in each lot the refrigerated rations were fed for the last 24 days. In addition, a vitamin mixture equivalent to the amount already present in the ration was added to the evening feeding for each pig to insure against a possible vitamin deficiency. Both of the pigs that died were receiving the refrigerated ration for at least 7 days, and the extra vitamin supplement for at least 4 days before they died.

Inasmuch as it was impossible to attribute the cause of death to any of the rations fed, and since only two pigs out of 44 in two experiments were thus affected, the data obtained are believed to be valid. It is unfortunate that the data for the lot receiving 0.45% methionine, the fastest growing group, in trial B were for only three pigs; however, it is believed that the conclusions are justified, even with this limitation.

As the methionine plus cystine requirement of growing-fattening pigs is shown to be not more than 0.69% of the 22% protein ration containing soybean oil meal (Curtin et al., '52) and approximately 0.72% of the 22% protein ration containing isolated soybean protein, it is suggested that the requirement is approximately 0.7% of the ration containing 22% protein or 3.2% of the protein. Calculation shows the corresponding value reported by the Purdue workers (Shelton et al., '51) to be 2.86% of the protein which is 10% lower than the requirement as reported herein. The Purdue workers used a purified diet that required supplementation with 0.5% of a combination of DL-methionine and L-cystine, or DL-methionine alone for optimum growth. The crystalline amino acids might be expected to be more highly digestible than the amino acids present in natural feeds such as the soybean protein used in this study. Only 0.2% DL-methionine added to the isolated soybean protein-brewers' dried yeast ration was necessary for optimum performance in the studies reported herein. The slightly lower digestibility of methionine and cystine present in natural feeds,

or a difference in the pigs used, might account for the difference in the requirement found in this study and that reported by the Purdue workers.

The isolated soybean protein rations contained 0.26% and 0.27% cystine. Earlier work (Curtin et al., '52) has indicated that 0.38% cystine probably can replace a corresponding amount of methionine. Previously, Shelton et al. ('51) found that L-cystine could replace at least 50% of the methionine requirement of the pig.

SUMMARY

Two experiments were conducted to study the methionine requirement of weanling pigs fed a ration containing 22% protein from isolated soybean protein and brewers' dried yeast. From the data obtained, the methionine plus cystine requirement for weanling pigs appears to be approximately 0.7% of the ration containing 22% protein. This corresponds to 3.2% of the protein. Cystine apparently can be used to replace methionine in the ration up to at least 1.7% of the protein.

ACKNOWLEDGMENTS

These studies were supported in part by a grant from the Herman Frasch Foundation. The cystine analysis of the isolated soybean protein was made by Dr. R. J. Block, New York Medical School, New York, N. Y.; the vitamin B_{12} was supplied by Merck and Co., Inc., Rahway, N. J.; and the aureomycin hydrochloride by Lederle Laboratories, Pearl River, N. Y.

LITERATURE CITED

BELL, J. M., H. H. WILLIAMS, J. K. LOOSLI AND L. A. MAYNARD 1950 The effect of methionine supplementation of a soybean oil meal-purified ration for young pigs. J. Nutrition, *40:* 551.

BLOCK, R. J., AND H. H. MITCHELL 1946–47 The correlation of the amino acid composition of proteins with their nutritive value. Nutrition Abstr. and Rev., *16:* 249.

CURTIN, L. V., J. K. LOOSLI, J. P. WILLMAN AND H. H. WILLIAMS 1952 Methionine as a supplement to soybean oil meal for weanling pigs. J. Animal Sci., *11:* 459.

DYER, I. A., J. L. KRIDER AND W. E. CARROLL 1949 Known and unidentified factors supplement a corn-soybean meal ration for weanling pigs in dry lot. Ibid., *8:* 541.

GRAU, C. R., AND H. J. ALMQUIST 1943 The utilization of the sulfur amino acids by the chick. J. Nutrition, *26:* 631.

LYMAN, R. L., AND C. A. ELVEHJEM 1951 The lability of thiamine in certain purified rations. Proc. Soc. Exp. Biol. Med., *77:* 813.

MILLER, R. F. 1951 Differentiation of unidentified factors required by the chick. Ph.D. Thesis, Cornell University, Ithaca, New York.

NATIONAL RESEARCH COUNCIL 1950 Recommended nutrient allowances for domestic animals, no. II. Washington, D. C.

SHELTON, D. C., W. M. BEESON AND E. T. MERTZ 1951 The effect of methionine and cystine on the growth of weanling pigs. J. Animal Sci., *10:* 57.

THE RELATION BETWEEN VITAMIN B_{12} DEFICIENCY AND LACTATION IN RATS FED PURIFIED CASEIN RATIONS

LESLIE P. DRYDEN, ARTHUR M. HARTMAN AND CHARLES A. CARY[1]

Bureau of Dairy Industry, Agricultural Research Administration, U. S. Department of Agriculture, Washington, D. C.

ONE FIGURE

(Received for publication July 28, 1952)

It has been reported from this laboratory and elsewhere that the weaning weights of young rats nursed by mothers on vitamin B_{12}-deficient rations were suboptimal (Zucker et al., '48; Dryden, Hartman and Cary, '49; Emerson, Kummer and Zanetti, '49; Lepkovsky et al., '50, '51; Nell and Phillips, '50; Watts et al., '50) and that their post-weaning weight gains were likewise less than when the young had been nursed by mothers on rations containing vitamin B_{12} or B_{12}-active materials (Cary and Hartman, '43–'47; Hartman et al., '49; Emerson et al., '50). Lepkovsky et al. ('51) reported that the impaired lactation observed with first litter young of mothers transferred at positive mating to a vegetable protein B_{12}-deficient diet was apparently the result of an inadequate amount of milk rather than a milk of poor quality, but they attributed this condition to unknown factors inherent in their vitamin B_{12}-deficient ration rather than to a vitamin B_{12} deficiency as the major cause.

In this paper the feeding of certain vitamin B_{12}-deficient casein-containing rations to the mother is considered in rela-

[1] Formerly Head, Division of Nutrition and Physiology; retired Feb., 1950.

tion to its effect upon the young during the pre-weaning and post-weaning period, and consideration is given to the manner in which B_{12} deficiency affects lactation. Survival during the pre-weaning period of young born to mothers on these rations has been discussed in a previous paper (Dryden, Hartman and Cary, '52).

TABLE 1

Composition of rations [1]

	RATION NUMBER							
	47	192	194	260	262	300	302	443
	%	%	%	%	%	%	%	%
Dextrin [2]	45.50	54.85	45.28	30.50	40.38	40.32	54.32	53.43
Lactose (U.S.P. XIII)	..	..	..	15.00	15.00	15.00	..	1.50
Casein (10x hot alcohol extd.) [3]	20.00	30.00	20.00	20.00	20.00	30.00	30.00	30.00
DL-methionine		0.20	0.20	..	..	..	..	0.20
Yeast, dried brewers' [4]	20.00	..	20.00	20.00	10.00	..	..	..
Salts (Hawk and Oser, '31)	4.50	4.50	4.50	4.50	4.50	4.50	4.50	4.50
Succinylsulfathiazole					..	..	1.00	
Cottonseed oil	9.85	9.85	9.85	9.85	9.85	9.85	9.85	9.85
Fish liver oil [5]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Vitamins added		0.46	0.03		0.12	0.18	0.18	0.37
Vitamins: mg/100 gm ration								
Thiamin hydrochloride	..	1.6	..	..	0.5	0.8	0.8	1.6
Riboflavin	..	1.6	..	..	0.5	0.8	0.8	1.6
Pyridoxine hydrochloride	..	1.6	..	..	0.5	0.8	0.8	1.6
Calcium pantothenate	..	20.0	..	..	3.1	5.0	5.0	10.0
Choline chloride	..	240.0	..	..	75.0	120.0	120.0	240.0
Nicotinic acid	..	20.0	..	..	3.1	5.0	5.0	10.0
Inositol	..	20.0	..	..	3.1	5.0	5.0	10.0
Para-aminobenzoic acid	..	120.0	..	..	18.8	30.0	30.0	60.0
Biotin	..	0.01	..	..	0.006	0.01	0.01	0.02
Pteroylglutamic acid [6]		0.20	..	..	0.06	0.10	0.10	0.20
Ascorbic acid	..	10.0	10.0	..	3.08	4.86	4.86	10.0
Alpha-tocopherol acetate	..	20.0	20.0	..	6.25	9.72	9.72	20.0
2-methyl, 1, 4-naphthoquinone		0.5	0.5	..	0.16	0.25	0.25	0.5

[1] Ration 45 was the same as ration 47, and ration 197 was the same as ration 194 except that in each case 10% dextrin replaced 10% of yeast. Rations 195, 301 and 303 were the same as rations 192, 300 and 302, respectively, except that in each case 5% dextrin replaced 5% casein.

[2] "Amidex," a dextrinized cornstarch manufactured by the Corn Products Refining Co., New York, N. Y.

[3] For preparation of casein, see Hartman, Dryden and Cary ('51).

[4] Strain G, Anheuser-Busch Co., St. Louis, Mo.

[5] "Navitol with Viosterol (concentrated oleovitamin A and D) U.S.P.," E. R. Squibb and Sons, New York, N. Y. According to manufacturer, contained per gram 65,000 I.U. of vitamin A and 13,000 I.U. of vitamin D.

[6] Kindly supplied by Dr. E. L. R. Stokstad and Dr. T. H. Jukes, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

EXPERIMENTAL PROCEDURE

The composition of the B_{12}-deficient rations used in these experiments is described in table 1; all of them are essentially modifications of ration 47 as indicated in the table. All of the rations and the distilled water were supplied ad libitum. Except as otherwise indicated, supplements of 15 units/ml APA liver extract [2] or crystalline vitamin B_{12} [3] were administered orally, either daily or three times per week.

The mothers used in these experiments were of 4 types: (1) those fed the stock colony ration [4]; (2) those transferred at parturition or a day or so thereafter from the stock colony ration and maintained on the experimental ration during lactation; (3) those continued on the experimental ration beyond the initial lactation described in 2; and (4) rats whose ancestors had been maintained on the experimental ration for one or more generations. During lactation, the mothers and their young were bedded on wood shavings; beyond 28 days of age, the young were kept in individual cages on raised screen floors.

Litters born while the mothers were on the stock ration and litters in the "generation" experiment shown in table 4 were reduced to 6 in number at birth; all of the others were reduced to 6 at the end of one week. The young that were retained were weaned at 25 days of age.

RESULTS AND DISCUSSION

Pre-weaning weights of young

The effect of vitamin B_{12} deficiency upon the weights of young rats nursed by mothers on alcohol-extracted casein rations is shown in table 2 for various ages during the lactation period. It can be seen that, although differences in body

[2] Lederle.

[3] The crystalline vitamin B_{12} was kindly supplied by Dr. D. F. Green and Dr. M. A. Schooley, Merck and Co., Inc., Rahway, N. J.

[4] Composition of stock ration: yellow corn meal 69.5; linseed oil meal 14.0; meat scrap 9.0; casein (commercial) 4.0; alfalfa meal 2.0; bone meal 2.0; sodium chloride 0.5. Supplements of lettuce and carrots were fed once a week.

weight of the young occurred between B_{12}-deficient and B_{12}-supplemented rations in all cases before weaning, there was considerable variation in the time when a significant difference [5] could first be observed. Thus, of the young born to mothers transferred at parturition from the stock ration to the experimental rations (groups 1, 3, 5, 7, 9), significant differences in weight as early as 14 days occurred between rats on the B_{12}-deficient ration and those on the B_{12}-supplemented ration in only two of the 5 groups — with the male young on the lactose-containing yeast-free ration (300) and with both male and female young on one of the yeast rations (194). By 21 days of age, however, with the exception of one small group, significant differences in weight were observed with all groups of animals on all rations except 192. It has been shown in a previous publication (Hartman et al., '51) that feeding ration 192 was often conducive to the intestinal synthesis of vitamin B_{12}-active material but even here, significant differences in weight between the young from B_{12}-supplemented mothers and those from B_{12}-deficient mothers could be observed by the 25th day of age.

The percentage increase in weight of the young from the B_{12}-supplemented mothers above that of the young from the B_{12}-deficient mothers also varied in a similar manner from ration to ration at the various ages. Thus at 25 days of age, it ranged from 5% to 36%, being highest on ration 300 and lowest on ration 192.

Because of the poor survival during the first few days after birth of the offspring of mothers maintained on the yeast-free B_{12}-deficient rations through additional gestations and lactations beyond the initial nursing period, comparisons of pre-weaning weights between the young from B_{12}-deficient and those from B_{12}-supplemented mothers (groups 2, 4, 6, 8)

[5] The symbol ** adjacent to or in connection with a t or F value indicates statistical significance at or less than the 1% level; * indicates significance at the 5% level or between the 5% and 1% levels; no * indicates no statistically significant difference.

are limited in some cases to very small numbers. It would appear, however, that in most cases during these later lactations, significant differences occurred at an earlier age and were greater in magnitude than they had been during the initial lactation on the experimental rations. Thus the body weights at 25 days of age of the young from B_{12}-supplemented mothers were 14 to 55% greater than those of the young from B_{12}-deficient mothers, with the greatest differences occurring on ration 300 and the smallest on ration 192 as before.

Change in weight of the mother during lactation

The change in weight of the mother during the nursing period is also shown in table 2. There was no significant difference in any group between the changes in weight of the B_{12}-deficient and B_{12}-supplemented mothers. After the mothers were maintained on the experimental rations through successive gestation and lactation periods, there was a tendency for both unsupplemented and supplemented mothers to lose more weight than they did during the lactation immediately following the transfer to the experimental ration; this tendency was highly significant ($t = 5.6^{**}$) with the supplemented mothers.

Post-weaning weight gains

The effect of feeding a supplement of vitamin B_{12} to mothers placed on a B_{12}-deficient ration is reflected not only in the weights of the young preceding weaning but also in their post-weaning weight gains. A comparison of such weight gains is shown in table 3 between the young whose mothers were put on a B_{12}-deficient ration at parturition and those whose mothers were fed either the stock ration or a B_{12}-supplemented ration during lactation. (Similar effects were obtained whether the mothers were continued on the stock ration or put on the deficient ration supplemented with either APA liver extract or crystalline vitamin B_{12}.)

TABLE 2

Effect of vitamin B_{12} deficiency upon body weights of young and changes in weight of mother during lactation

GROUP	RATION OF MOTHER DURING GESTATION	RATION OF MOTHER DURING LACTATION	SUPPLEMENT [1] FED TO MOTHER	NO. OF MOTHERS	NO. OF YOUNG AT 25 DAYS OF AGE [2]		BODY WEIGHT OF YOUNG AT:												AVERAGE CHANGE IN WT. OF MOTHERS DURING LACTATION	
					Males	Females	7 days of age [3]	14 days of age				21 days of age				25 days of age				
								Males		Females		Males		Females		Males		Females		
							gm.	gm.	t	gm.	t	gm.	t	gm.	t	gm.	t	gm.	t	gm.	t
1	Stock	192	None	13	47	29	16	29		30		46		46		60		58		— 2.6	
									1.3		0.0		1.2		0.7		3.9**		2.2*		0.6
	Stock	192	B_{12}	17	50	45	16	30		30		47		46		65		61		— 5.5	
2	195	192	None	5	14	13	12	25		26		40		40		51		50		— 17.2	
									4.0**		2.3*		3.8**		2.9**		7.9**		3.9**		0.2
	195	192	B_{12}	13	55	48	15	30		29		47		44		64		57		— 16.1	
3	Stock	300	None	5	26	3	13	23		23		34		33		46		42		— 3.4	
									2.5*		1.6		6.0**		6.2**		6.5**		6.8**		1.2
	Stock	300	B_{12}	5	19	10	14	25		26		41		42		59		57		+ 8.4	
4	301	300	None	1	3	3	12	22		24		33		37		44		45		— 42.0	
									5.6**		7.2**		8.7**		10.1**		9.2**		10.9**		..
	301	300	B_{12}	3	22	17	17	32		31		51		50		68		65		— 17.7	
5	Stock	302	None	5	25	5	13	24		24		37		37		48		47		— 3.0	
									0.8		0.7		2.5*		1.1		5.1**		2.0		1.0
	Stock	302	B_{12}	4	18	6	13	25		25		40		40		55		53		— 8.2	
6	303	302	None	1	2	2	16	28		27		38		38		43		42		— 1.0	
									0.1		1.5		1.0		0.3		2.9**		6.6**		..
	303	302	B_{12}	4	20	18	12	27		25		42		39		55		51		— 20.6	
7	Stock	194	None	10	39	19	15	25		24		39		38		51		48		— 3.1	
									4.4**		4.1**		5.6**		4.0**		10.4**		6.9**		2.1
	Stock	194	B_{12}	10	50	10	14	27		27		43		44		60		59		+ 7.3	
8	197	194	None	7	38	24	14	25		23		38		35		49		46		— 1.2	
									5.6**		5.7**		6.4**		5.3**		6.4**		6.4**		1.2
	197	194	B_{12}	9	38	38	15	30		29		48		46		62		60		— 7.1	
9	Stock	47	None	10	35	17	17	31		29		46		41		59		52		— 0.8	
									1.4		0.7		3.0**		2.4*		3.8**		3.0**		1.3
	Stock	47	Liv. ext.	10	38	17	16	32		30		51		47		66		60		+ 11.8	

[1] Crystalline vitamin B_{12} — 1 or 2 μg/day; 15 units/ml APA liver extract — 0.10 ml/day.
[2] Some groups contained slightly larger numbers at earlier ages.
[3] At 7 days of age, males and females were not weighed separately.

When the mothers were fed a B_{12}-containing ration, the 4-week gains in weight of the young subsequent to 28 days of age were 41 to 57% greater for the males, and 30 to 31% greater for the females when the young were fed a source of

TABLE 3

Effect of maternal ration during lactation upon post-weaning weight gains of young

GROUP	RATION OF MOTHER DURING LACTATION [1]	SUPPL. TO MOTHER'S RATION [2]	RATION OF YOUNG AFTER 28 DAYS OF AGE	NO. OF LITTER-MATE PAIRS OF YOUNG	4-WEEK POST-WEANING WEIGHT GAINS [3]				
					No supplement		Vitamin B_{12} [4]		t
					gm	t	gm	t	
1 (males)	47	None	45	349	88	6.8**	154	2.3*	44.4**
	Stock	None	45	18	114		164		9.2**
	Stock	None	Stock	10	157		...		
2 (females)	47	None	45	81	57	8.8**	103	3.6**	22.8**
	Stock	None	45	14	87		114		6.6**
	Stock	None	Stock	15 [5]	98		...		
3 (males)	47	None	45	26	85	3.7**	160 [6]	0.1	12.9**
	47	Liv. ext.	45	26	108		161 [6]		6.1**
4 (females)	47	None	45	11	56	4.6**	104 [8]	0.7	6.9**
	47	Liv. ext.	45	11	76 [7]		99 [8]		5.0**
5 (males)	302	None	303	5	68	2.3	126	3.3*	6.4**
	302	B_{12}	303	4	96		151		4.3*
6 (males)	300	None	301	5	53	3.2*	146	1.2	8.1**
	300	B_{12}	301	4	93		131		2.8

[1] Fed to young until about 28 days of age.

[2] One-tenth ml/day, 15 units/ml APA liver extract or 1 μg/day crystalline B_{12} as indicated.

[3] Twenty-eight to 56 days of age.

[4] Liver extract: 0.02–0.10 ml/day (group 1); 0.02–0.05 ml/day (group 2); 0.02 ml/day (groups 3, 4). Crystalline B_{12}: 2 μg/day (groups 5, 6).

[5] Weaned at 21 days of age.

[6] Twelve rats.

[7] Ten rats.

[8] Six rats.

B_{12} than when they were given the B_{12}-deficient ration alone. On the other hand, when the mothers were maintained on an unsupplemented B_{12}-deficient ration during the nursing period, the 4-week post-weaning weight gains of the young fed a sup-

plement of B_{12}-active material were for two of the rations (45, 303) 75 to 88% greater, and for the other ration (301) 175% greater than those of the young fed the B_{12}-deficient ration alone.

The smaller differences between the young fed B_{12}-deficient and B_{12}-supplemented rations which were obtained when the mothers were fed a B_{12}-active ration were apparently due primarily to increased weight gains of the B_{12}-deficient young. Feeding B_{12} to the mother apparently had much less effect on the 4-week post-weaning weight gains of the young when the latter also received a source of B_{12}. Under these conditions, only three of the 6 groups showed a significant difference, the young from mothers who had received B_{12} having higher weight gains in these instances. Two of the groups in which no significant differences were observed were the only groups in which the comparisons were made directly between litter mates.

B_{12}-deficient rations over several generations

The 28-day weights and the subsequent 4-week post-weaning weight gains of the young maintained on yeast-containing B_{12}-deficient rations over several generations are shown in table 4. The parent generation mothers were transferred from the stock ration to the experimental ration at parturition of a litter, and carried through several matings on this ration. Their young born on the deficient ration were divided into two groups at 28 days of age, one maintained on a similar B_{12}-deficient ration and the other (litter mates of the first group) on the same ration plus a supplement of liver extract. These two groups were carried through the F_2 and F_3 generations.

The 28-day weight differences between the young from supplemented and unsupplemented mothers were much the same for both sexes in all generations (21 to 26% greater for the groups from supplemented mothers) except in the

TABLE 4

Changes in rate of growth of young as a result of keeping mothers on vitamin B_{12}-deficient yeast-containing rations over several generations

GENERATION OF MOTHERS	RATION OF MOTHERS DURING LACTATION		MALE YOUNG			FEMALE YOUNG			CHANGE IN WT. OF MOTHERS DURING LACTATION
	No.[1]	Suppl.	28-day wt.	4-week post-weaning weight gains		28-day wt.	4-week post-weaning weight gains		
				No. suppl.	Liv. ext.[2]		No. suppl.	Liv. ext.[2]	
			gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Parent[3]	47	None	63 ± 2.1[4] (29)[5]	79 ± 3.0 (17)	155 ± 5.6 (12)	61 ± 2.0 (29)	61 ± 1.8 (16)	93 ± 1.9 (13)	−5 ± 1.6 (12)[6]
F_1	47	None	57 ± 0.9 (75)	80 ± 2.6 (31)	129 ± 3.2 (24)	54 ± 0.8 (77)	54 ± 2.8 (42)	92 ± 2.8 (20)	−13 ± 2.8 (31)
F_1	47	Liv. ext.[2]	70 ± 1.0 (85)	122 ± 3.5 (42)	139 ± 3.9 (32)	66 ± 0.7 (92)	79 ± 1.8 (43)	93 ± 1.9 (38)	−15 ± 2.4 (34)
F_2	47	None	44 ± 2.1 (33)	67 ± 4.1 (14)	121 ± 4.4 (9)	40 ± 2.2 (23)	52 ± 2.5 (16)	89 ± 2.8 (5)	+1 ± 4.2 (10)
(to 2/1/47)	47	Liv. ext.[2]	72 ± 1.8 (14)	124 ± 6.7 (8)	149 ± 6.7 (6)	64 ± 1.3 (16)	80 ± 3.1 (8)	86 ± 5.7 (7)	−17 ± 5.3 (7)
F_2	47	None	62 ± 1.0 (155)	96 ± 3.8 (41)	138 ± 2.9 (32)	56 ± 0.9 (106)	63 ± 2.0 (31)	89 ± 2.4 (18)	−9 ± 1.9 (51)
(2/1/47 and later)	47	Liv. ext.[2]	75 ± 1.5 (69)	132 ± 4.7 (16)	142 ± 6.1 (13)	68 ± 1.5 (53)	86 ± 2.2 (14)	93 ± 3.6 (9)	−19 ± 3.7 (24)
F_3	47	None	62 ± 1.2 (79)	96 ± 11.3 (8)	148 ± 6.0 (8)	58 ± 1.0 (95)	69 ± 3.3 (8)	96 ± 5.9 (6)	−11 ± 3.1 (41)
F_3	47	Liv. ext.[2]	77 ± 1.1 (42)	.....	.....	73 ± 1.3 (19)	.....	.....	−17 ± 3.9 (11)

[1] Also fed to young until 28 days of age or thereabouts; young received ration 45 from that time on.

[2] Five-hundredths ml/day, 15 units/ml APA liver extract.

[3] Figures include only young born while mothers were on experimental rations.

[4] Standard error of the mean.

[5] Figures in parentheses indicate the number of young.

[6] Figures in parentheses indicate the number of litters.

early stage of the F_2 generation, where the average weight was 64% greater for the males and 60% greater for the females nursed by supplemented mothers.

If the 4-week post-weaning weight gains of the young from B_{12}-deficient mothers are considered, it can be seen that there was a significant tendency for those of both the unsupplemented and B_{12}-fed young to drop to a low point during the early stage of the F_2 generation, and after that for the B_{12}-fed young to regain the position held at the beginning of the experiment, and for the unsupplemented young to attain weight gains considerably in excess of those at the beginning of the experiment. This tendency is not pronounced in the young from the B_{12}-fed mothers.

It will be noted again that the unsupplemented young from mothers fed liver extract gained considerably more weight in the post-weaning period than did the young from mothers not fed liver extract. It is also evident that in these later generations, if the young were fed liver extract, the feeding of liver extract to the mothers had no effect on the post-weaning gains of the female young, but did have a significant effect in the case of the male young in the F_1 and early F_2 generations.

That the decrease in weight gains in some groups during the early part of the experiment might represent a gradual depletion of some factor not found in liver extract seems quite doubtful since the average weight gains of the supplemented young that came from mothers receiving liver extract were not significantly lower in the F_2 generation than in the parent generation.

The differences in gains in weight between the unsupplemented young from unsupplemented mothers and the supplemented young from supplemented mothers for the early part of the experiment were not markedly different from those found for the young from mothers put on the deficient rations at parturition (table 3, groups 3 and 4); however, the differences

in the latter part of the experiment were smaller. To a large extent these smaller differences were due to the greater growth of the unsupplemented young from the unsupplemented mothers. Thus, of such young in the late F_2 and the F_3 generations, 52% grew more than 100 gm, and 18.5% grew more than 120 gm while none of their ancestors born to the parent generation mothers grew over 100 gm for the 4-week period. There were, moreover, in this later period some growths (such as 146 gm and 162 gm) that were as high as the average for the young receiving the liver extract; such growths would seem to represent intestinal synthesis of B_{12}-active material, something that apparently did not occur in the earlier generations. It should be noted, however, that, considering all of the young in our colony that were reared by mothers transferred from the stock ration to B_{12}-deficient ration 47 in the previous two years, 21% of those growing during the same period of the year as the young belonging to the latter part of the F_2 generation and the F_3 generation in this experiment gained 120 gm or more during the 4-week post-weaning period.

It would appear then that retaining animals over several generations on these B_{12}-deficient yeast-containing casein rations did not produce effects on lactation that were very much more pronounced than those that were observed either in the lactations in the parent generation, if comparative weights during the lactation period are considered, or even in the lactation immediately subsequent to placing the mothers on the deficient ration at parturition, if the criterion used is the subsequent post-weaning weight gains.

Storage of vitamin B_{12} in the young

The effect of placing mothers on a B_{12}-deficient ration at parturition is shown not only in the pre-weaning weights and post-weaning weight gains but also in the storage of vitamin B_{12} in the bodies of the young. Lewis, Register and Elvehjem ('49) showed that, of the organs and tissues tested, the site of the greatest B_{12} concentration in the body of the rat was the

kidney and that the liver also contained appreciable B_{12} when this vitamin was included in the diet. Richardson et al. ('51) showed that the amount of B_{12} in the livers of 28-day-old rats was increased when a source of B_{12} had been included in the diet of the mother, but, since the diet presumably had also been available to the young, it is not clear from the data presented whether the increase occurred through a change in the B_{12} intake from the milk of the mother or through an increased intake from the diet by the young themselves. It seemed desirable therefore to run a similar test on rats not old enough to be taking in solid food.

For this experiment, test organs were obtained from young rats at 13 to 15 days of age. These young came from stock mothers who had been placed at parturition on ration 260 either with or without an oral supplement of crystalline B_{12} (2 μg/day); no significant differences in the weight of the test young or in the weights of their kidneys or livers were observed at the time they were sacrificed. The organs were assayed by a rat-growth assay method involving the use of 38- to 45-day-old B_{12}-deficient rats fed ration 262 and reared by mothers fed ration 260 during lactation. Each animal receiving test organs was given either the liver or both kidneys of one young rat per day for 12 days during the 14-day test period.

The assay indicated that the kidneys of the young from the mothers fed the B_{12}-deficient ration alone contained only about 56% ($t = 1.5$) as great a concentration of B_{12} as did the kidneys of the young from the mothers receiving a B_{12} supplement, while the livers contained only about 26% as much ($t = 2.9^*$). (This more rapid depletion of the liver is in agreement with the findings of Lewis et al., '49.) These differences occurred at a time when there could be no possibility of the young having consumed food other than their mother's milk.

Influence of access of young to feces of mother

Since the young from mothers fed a supplement of vitamin B_{12} in addition to the B_{12}-deficient rations grew better than

those from the mothers on the B_{12}-deficient rations alone, it was evident that the young themselves must have been receiving a supply of B_{12} when it was fed to the mothers. The experimental technique used in feeding the supplement to the mothers, that is, by means of a hypodermic syringe with the blunted needle placed well back in the throat or, more frequently, directly into the esophagus itself, would seem to preclude the possibility of the young securing any of the B_{12} from about the mouth and lips of the mother. It is not clear, however, that the B_{12} was necessarily secured by the young through the milk, since it would appear possible that some might be obtained from the mother's excreta.

Chow et al. ('51), Barbee and Johnson ('51) and Yamamoto et al. ('51) showed that when B_{12} was administered orally to rats, excretion of the vitamin occurred primarily through the feces. It has been observed in this laboratory that B_{12}-active material is contained in the feces of rats receiving oral supplements of the vitamin, even at suboptimal doses, but not in the feces of rats fed such unsupplemented B_{12}-deficient rations as 47 and 262. While it would not seem that consumption of the excreta could explain weight differences occurring before the young were consuming solid food, yet such consumption would present a reasonable explanation where differences did not occur until 21 days of age or later.

The rats whose pre-weaning weights are shown in table 2 (ration 47) and whose post-weaning weight gains are shown in table 3 (groups 3 and 4) were used to test this possibility. In this experiment, mother rats were used in pairs, both members of a pair having littered on the same day or not more than one day apart. The litters of each pair were interchanged between the two mothers so that each mother of the pair nursed the same number of young (5 or 6), and so that all of the young reared by one mother were composed of the same number of siblings of the same sex as those reared by the other mother of the pair. Ten pairs of mothers were treated in this way. One mother of each pair received a supplement of liver extract (0.10 ml/day) throughout the nursing

period while the other mother received no supplement. It will be noted that differences in the average body weights of the young nursed to these two groups of mothers first became significant at 21 days of age and that during the 28th to 56th day of life, the male young from the supplemented mothers,

TABLE 5

The influence of access to feces from rats fed liver extract upon the comparative weight gains of young nursed to mothers fed a B_{12}-deficient ration either with or without a supplement of B_{12}-active material

AGE	AVERAGE WEIGHT DIFFERENCES BETWEEN YOUNG NURSED TO MOTHERS FED RATION 45 ALONE AND THOSE FROM MOTHERS FED RATION 45 + LIVER EXTRACT [1]				
	Males			Females	
	Cages not interchanged	Cages interchanged		Cages not interchanged	Cages interchanged
days	*gm*	*gm*	*t*	*gm*	*gm*
	Body weights				
	(18 pairs)[2]	(21 pairs)		(4 pairs)	(13 pairs)
0–1	— 0.1	— 0.1	..	— 0.2	— 0.2
7	— 0.8	0.9	..	— 3.3	— 0.1
14	0.2	2.5	..	— 0.7	1.6
21	3.6	5.2	..	3.3	5.9
25	6.4	7.0	..	5.9	9.2
28	10.0	9.3	0.2	9.2	13.4
	Weight gains from 28 days of age				
	(13 pairs)	(13 pairs)		(2 pairs)	(9 pairs)
35	8.1	3.1	1.3	7.5	9.3
42	17.1	10.9	0.9	4.5	14.2
49	22.5	16.9	0.6	13.0	15.9
56	25.2	19.4	0.5	25.0	18.8

[1] Differences are designated positive where weights or weight gains of young nursed to mothers receiving liver extract are higher, and negative where lower.

[2] Only 17 young in liver extract group.

although receiving no supplement themselves, grew 27% faster, and the female young 36% faster than the corresponding young from the unsupplemented mothers.

In order to determine whether this extra growth could be due in some measure to B_{12} obtained from the excreta of the liver-fed mothers, the two mothers of a pair and the young nursing to them, had, in some pairs, their cages interchanged

daily beginning at different periods during the nursing period and continuing to the 28th day of age, so as to give the young with each mother and the mothers themselves as nearly equal access as possible to the excreta of the mother receiving the liver extract.[6]

The data as presented in tables 2 and 3 represented the combined data of those litters whose cages were and were not alternated. In table 5 the pairs of litters which had their cages interchanged are compared with those which did not have their cages interchanged. It can be seen that at no time during the experiment — either during lactation or during the succeeding 4-week period — were the differences in weights or weight gains between the young nursed by supplemented mothers and those nursed by unsupplemented mothers significantly greater when the cages were not alternated than when they were alternated. It seems then that access to the excreta of the mother was not a significant factor in explaining the differences between the weight gains of the young from B_{12}-supplemented mothers and those from B_{12}-deficient mothers under our experimental conditions.

Quantity and quality of milk produced during lactation

Since it appears that young rats nursed to mothers fed a separate supplement of B_{12}-active material did not obtain a significant amount of B_{12} either from the ration or supplement fed the mothers or from the mother's excreta, it would appear that they must have obtained B_{12} through the mother's milk. This B_{12} could have been obtained through a larger amount of milk containing the same concentration of B_{12} as that given by the B_{12}-deficient mothers, through the same amount of milk containing a larger B_{12} concentration or through a larger supply containing an increased concentration.

It seemed that the injection of B_{12} into some of the young of a litter rather than feeding it to the mothers might throw

[6] The shavings upon which the mother and young were nested, together with the excreta present, were replaced by clean shavings at about weekly intervals during the 28-day period.

some light on this question, since if injected young grew no better than uninjected young nursed to the same B_{12}-deficient mother, it would appear that the quantity of the milk was being affected by feeding B_{12} to the mothers. The results of such an experiment are shown in figure 1. No differences in body weight could be observed between injected and non-injected young before they were eating solid food, and no sig-

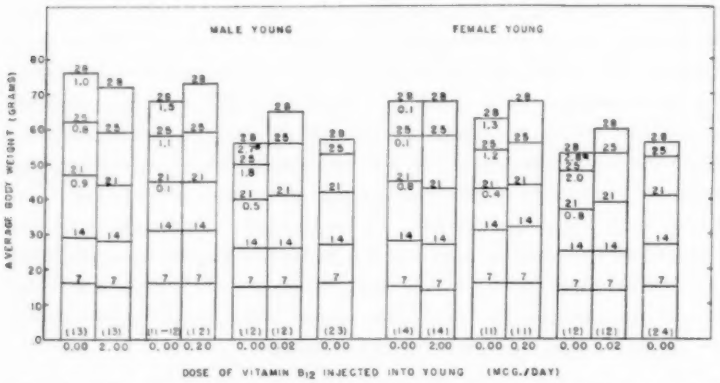
MALE YOUNG
FEMALE YOUNG
AVERAGE BODY WEIGHT (GRAMS)
DOSE OF VITAMIN B12 INJECTED INTO YOUNG (MCG./DAY)
0.00 2.00 0.00 0.20 0.00 0.02 0.00 0.00 2.00 0.00 0.20 0.00 0.02 0.00

Fig. 1 Effect on pre-weaning weights of vitamin B_{12} injected into young nursed to mothers on B_{12}-deficient rations. Figures above lines indicate age of young (in days); figures below lines indicate *t* values for difference in weight between non-injected and injected young. Figures in parentheses at the bottom of each column indicate the number of young in each group. The young were derived from 8 or 9 mothers at each dose level. The doses shown are average daily doses; injections were actually made on the 4th, 8th, 12th, 16th, 20th and 22nd days of age.

nificant differences were noted until 28 days of age. Even then, a significant difference was obtained only when a sub-optimal dose was injected.

At the two higher doses, it appeared that some B_{12} was being transferred from the injected young to the non-injected young. Chow et al. ('50) and Barbee and Johnson ('51) showed that when B_{12} was injected into rats, excretion of the vitamin occurred primarily through the urine. In the present experiment, since it appeared that no B_{12} could have been secured

by the mother or uninjected young from the injection site, it would seem probable that the mother obtained some B_{12} in the process of cleaning the young and then transferred some of it through her milk to the uninjected young. The results indicate that this was not a significant factor at the suboptimal dose injected.

The results of this experiment are in accord with the idea that the feeding of B_{12} to the mother affected the quantity rather than the quality of milk produced by the mothers.

Since it would appear that the change in weight of the young after a short period of nursing would reflect primarily the amount of milk secured by the young from the mother, this indirect method was used in an effort to obtain some measure of the relative output of milk by B_{12}-deficient and B_{12}-supplemented mothers. Stock colony mothers were taken at parturition in pairs, both members of the pair having given birth to litters on the same day or not more than one day apart. They were placed on a B_{12}-deficient ration (260), one member of the pair receiving an oral supplement of crystalline B_{12} (2 μg/day) and the other receiving no supplement. At 11 to 13 days of age, the young of both members of the pair were separated from their mothers in the late afternoon. The next morning the young were returned to the mothers, three of the young from the supplemented mother and three from the unsupplemented mother being given to each member of the pair to nurse. The weight changes of the young over the next 5 hours were then determined. The results are shown in table 6.

It appeared that the supplemented mothers were producing at this time about 20% more milk on the average than the unsupplemented mothers. Analysis of variance indicated that the difference in intake by the young occurred primarily between the two groups of the young previously reared by the supplemented mothers ("positive" young); only a small and non-significant difference occurred between the groups that were previously reared by the non-supplemented mothers ("negative" young). However, the difference between the "positive" young and the "negative" young was significant

only when both groups were nursed to supplemented mothers. Interpretation of the data is complicated somewhat since in each case a comparison must be made between the mother's own young and the foster young nursed by her; however, a similar experiment involving only supplemented mothers showed no significant difference between the amount of milk secured by a mother's own young and the foster young nursed

TABLE 6

Effect of vitamin B_{12} deficiency during lactation on quantity of milk produced [1]

	AVERAGE 5-HOUR WT. CHANGE PER NURSING YOUNG			
	"Negative" young [2]	"Positive" young [2]	All young	Analysis of variance *F* young
	gm	*gm*	*gm*	
Mothers fed no supplement	1.22	1.12	1.17	Not significant
Mothers fed vit. B_{12}	1.28	1.51	1.40	Significant*
Analysis of variance: *F* mothers	Not significant	Significant**	Significant**	

[1] Seven pairs of mothers and 84 young were used in this experiment. At the time of the test, the young were 12 to 14 days of age and their average body weights were 25.4 gm for the young from the unsupplemented mothers and 25.6 gm for the young from the supplemented mothers. An 8th pair of mothers has been omitted since one mother of the pair did not nurse the young to any appreciable extent when they were given to her.

[2] "Negative" young are those previously reared by unsupplemented mothers; "positive" young are those previously reared by supplemented mothers.

to her when both mothers had received the same amount of vitamin B_{12}. Thus it would appear not only that the B_{12}-deficient mothers produced less milk but that there was less inclination for their young to take in additional milk when it was available.

While the evidence indicates that the B_{12}-supplemented mothers produced more milk than the B_{12}-deficient mothers, this does not necessarily mean that the milk produced by the

supplemented mothers did not also contain a greater concentration of B_{12}. Meyer et al. ('51) did not find a significant increase in the B_{12} concentration (determined microbiologically) of rat milk when mother rats on purified casein rations were supplemented with 0.1 μg B_{12} per day; however, they did obtain such an increase when the rats were fed an all-plant ration supplemented with higher levels of B_{12}.

Evidence obtained in this laboratory has shown that with such B_{12}-deficient rations as 262 and 47, the weight differences between supplemented and unsupplemented young during the post-weaning period are accompanied by a decreased food consumption by the unsupplemented young; thus when the supplemented young were limited to the food intake of the unsupplemented young, no differences in weight gain were observed. It would seem that if the same situation prevails during lactation, differences in the quality of milk would not be reflected by differences in the weight of the young unless the quantity of milk was also affected. It thus appears that differences in body weights that occurred before the young were eating solid food may well have been caused entirely by changes in the quantity rather than in the quality of milk produced by the mothers, and further, that one factor bringing about the relative decrease in the quantity of milk produced by the deficient mother may have been the failure of the deficient young to nurse out all their mother's milk because of their limited appetite. However, since in the previously described experiment involving injected young no significant difference was observed in the weight gain between uninjected and injected young during the nursing period, it would seem that B_{12} deficiency must have also had a direct effect on the mothers in decreasing the total quantity of milk produced; had the effect of the B_{12} deficiency been only upon the appetite of the B_{12}-deficient young, the injected young would presumably have obtained more milk than the uninjected young and hence gained more weight.

Food consumption during lactation

Table 7 shows the food consumption of B_{12}-deficient and B_{12}-supplemented mothers and their litters during lactation on B_{12}-deficient rations 192 and 443; these two rations were

TABLE 7

Food consumption of mothers and litters during the lactation period on B_{12}-deficient and B_{12}-supplemented rations

RATION FED DURING LACTATION		NO. OF LITTERS [1]	FOOD CONSUMPTION DURING							
			1–7th day		8–14th day		15–21st day		22–25th day	
No.	Supplement		gm	t	gm	t	gm	t	gm	t
			Mothers transferred from stock to experimental ration at parturition							
192 or 443	None	10	20.8		31.2		39.3		49.3	
				0.1		0.6		0.7		1.4
192 or 443	B_{12} (2 μg/day)	11	20.9		32.0		40.9		53.0	
			Mothers on experimental ration through previous lactation period(s):							
192 or 443	None	7	15.9		26.3		34.5		37.4	
				4.2**		2.8*		2.2*		4.9**
192 or 443	B_{12} (2 μg/day)	13	19.5		29.8		39.0		48.9	

[1] All of the litters included here whose mothers were transferred to the experimental rations at parturition contained 6 young each throughout the lactation period. All those whose mothers had been on the experimental ration through previous lactation periods contained 6 young each from the end of the first week of lactation to weaning; during the first 7 days of lactation, an average of 7.90 young were nursed by each mother in the B_{12}-deficient group and an average of 7.92 young were nursed by each mother in the B_{12}-supplemented group.

very similar and the weights of the young at comparable periods during lactation were essentially the same. By comparison with table 2 (ration 192), it can be seen that differences in food consumption of unsupplemented and supple-

mented mothers largely followed differences in weights of the young during the lactation period. Thus it would appear that, as might be expected, a decreased output of milk by the mothers was accompanied by a decreased intake of food by the mothers.

SUMMARY

The feeding of vitamin B_{12}-deficient casein-containing rations to mother rats during the lactation period resulted in smaller pre-weaning weights and post-weaning weight gains of the young, and in a decreased storage of B_{12} in the organs of the young previous to the time that they began to eat solid food. Significantly lower pre-weaning weights of the young occurred more quickly when the B_{12}-deficient rations contained yeast than when they were yeast-free. A similar effect was noted when lactose-containing rations were compared to lactose-free rations. Retaining mothers over several generations on yeast-containing B_{12}-deficient rations, supplemented and unsupplemented with B_{12}, did not result in differences between pre-weaning weights of young or between post-weaning weight gains of young that were markedly greater than those that occurred in the parent generation.

Access to the B_{12}-fed mother's excreta was not found to be a significant factor in explaining differences in weights or weight gains between the young from B_{12}-deficient and those from B_{12}-supplemented mothers. It appeared that a decrease in the quantity of milk produced by B_{12}-deficient mothers was the primary factor in explaining the lower weight gains prior to the time the young began to consume solid food. The decreased milk output was accompanied by decreased food consumption by the deficient mothers.

LITERATURE CITED

BARBEE, K. W., AND B. C. JOHNSON 1951 Metabolism of radioactive vitamin B_{12} by the rat. Proc. Soc. Exp. Biol. Med., *76:* 720.

CARY, C. A., AND A. M. HARTMAN 1943–47 Unidentified nutrients. Yearbook of Agriculture, p. 779.

CHOW, B. F., L. BARROWS AND C. LANG 1950 The microbiological activity of vitamin B_{12} in the urine of normal rats following the oral and subcutaneous administration of this vitamin. J. Nutrition, *42:* 405.

CHOW, B. F., C. ROSENBLUM, R. H. SILBER, D. T. WOODBURY, R. YAMAMOTO AND C. A. LANG 1951 Oral administration of vitamin B_{12} containing cobalt^{60} to rats. Proc. Soc. Exp. Biol. Med., *76:* 393.

DRYDEN, L. P., A. M. HARTMAN AND C. A. CARY 1952 The effect of vitamin B_{12} deficiency upon the survival of young born to rats fed purified casein rations. J. Nutrition, *46:* 281.

———— 1949 The relation of vitamin B_{12} deficiency to ovarian function and reproduction in the rat. Abstr., 116th meeting, Am. Chem. Soc., p. 39A.

EMERSON, G. A., M. B. KUMMER AND M. E. ZANETTI 1949 Influence of vitamin B_{12} upon growth and reproduction in rats. Abstr., 116th meeting, Am. Chem. Soc., p. 36A.

———— 1950 The effect of vitamin B_{12} upon reproduction and growth. Abstr., 117th meeting, Am. Chem. Soc., p. 13A.

HARTMAN, A. M., L. P. DRYDEN AND C. A. CARY 1949 A chemically unidentified nutrient in milk and in non-fat milk solids. Proc. 12th Intern. Dairy Congr. (Stockholm), sect. 2, p. 156.

———— 1951 The effect of riboflavin on the bacterial synthesis of vitamin B_{12}-active material in the rat. Arch. Biochem. Biophys., *34:* 324.

HAWK, P. B., AND B. L. OSER 1931 A modification of the Osborne-Mendel salt mixture. Science, *74:* 369.

LEPKOVSKY, S., H. J. BORSON, R. BOUTHILET, R. PENCHARZ, D. SINGMAN, M. K. DIMICK AND R. ROBBINS 1951 Reproduction in vitamin B_{12}-deficient rats with emphasis upon intrauterine injury. Am. J. Physiol., *165:* 79.

LEPKOVSKY, S., H. J. BORSON, D. SINGMAN, R. PENCHARZ, R. BOUTHILET AND M. K. DIMICK 1950 The role of vitamin B_{12} in gestation, lactation and postlactation in rats. Abstr. 117th meeting, Am. Chem. Soc., p. 13A.

LEWIS, U. J., U. D. REGISTER AND C. A. ELVEHJEM 1949 Vitamin B_{12} content of various organs and tissues of the rat. Proc. Soc. Exp. Biol. Med., *71:* 509.

MEYER, M. L., H. T. THOMPSON AND C. A. ELVEHJEM 1951 The effect of vitamin B_{12} on reproduction and lactation in rats receiving pork or beef diets. J. Nutrition, *45:* 551.

NELL, R. E., AND P. H. PHILLIPS 1950 Contributions of brewers' yeast to a diet deficient in reproductive factors. Ibid., *42:* 117.

RICHARDSON, L. R., P. W. WITTEN AND J. R. COUCH 1951 Diet of mother and vitamin B_{12} content of tissues of infant rats. Proc. Soc. Exp. Biol. Med., *76:* 265.

WATTS, A. B., W. SWANK, R. J. OHMAN, O. B. ROSS AND R. W. MACVICAR 1950 The effect of supplementation of high corn diets on rat reproduction and lactation. J. Nutrition, *41:* 533.

YAMAMOTO, R., C. BARROWS, JR., C. LANG AND B. F. CHOW 1951 Further studies on the absorption of vitamin B_{12} following oral and parenteral administration. Ibid., *45:* 507.

ZUCKER, T. F., L. M. ZUCKER AND V. BABCOCK 1948 Lactation in rats on well fortified all-plant rations. Arch. Biochem., *19:* 323.

THE EFFECT OF DIET ON THE COMPARATIVE ACTIVITIES OF PYRIDOXAL, PYRIDOXAMINE AND PYRIDOXINE FOR CHICKS[1, 2, 3]

PAUL E. WAIBEL, W. W. CRAVENS AND ESMOND E. SNELL[4]
Departments of Poultry Husbandry and Biochemistry, University of Wisconsin, Madison

(Received for publication July 15, 1952)

Most of the vitamin B_6 in natural foodstuffs is present as pyridoxal or pyridoxamine rather than as pyridoxine (Rabinowitz and Snell, '48). Despite this fact, most nutritional studies with this vitamin have employed pyridoxine. For this reason, additional data on the comparative activities for higher animals of these naturally-occurring forms of the vitamin are needed.

Pyridoxal, pyridoxamine and pyridoxine are equally active for rats and chicks when administered by injection or by mouth apart from other components of the ration (Snell and Rannefeld, '45; Sarma et al., '46a). The injected compounds are also equally active for the dog (Sarma et al, '46b). When mixed in synthetic rations high in soluble carbohydrates, pyridoxal and pyridoxamine are distinctly less active than pyridoxine for chicks (Sarma et al., '46a), rats (Sarma et al., '46a; Linkswiler et al., '51) and mice (Miller and Baumann, '45). With rats, the addition of aureomycin to such rations reduces the requirement for each of the forms of vitamin

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[2] Supported in part by a grant from Merck and Co., Rahway, N. J.

[3] We are indebted to Merck and Co., for the vitamin B_{12}; to Lederle Laboratories, Pearl River, N. Y., for the folic acid; and to Wilson and Co., Chicago, Ill., for the gelatin.

[4] Present address: University of Texas, Austin, Texas.

B_6; however, the effect is greater with pyridoxal and pyridoxamine, so that under these conditions the three forms of the vitamin are equally active (Linkswiler et al., '51). Addition of substantial amounts of methionine to such rations increases the requirement of rats for vitamin B_6, and pyridoxal and pyridoxamine equal or surpass pyridoxine in growth-promoting activity under these conditions (DeBey et al., '52).

It is clear that the relative activities of the forms of vitamin B_6 vary with the ingredients of the ration. In the present study, the effects of variations in the carbohydrate of the diet and supplementation with aureomycin on their activity for chicks were determined.

EXPERIMENTAL

The chicks used in this study were the progeny of New Hampshire males and Single Comb White Leghorn females fed diet B-1 (Robblee et al., '48). Ten or more chicks were used per group. They were kept in electrically heated batteries with raised screen floors for the experimental period of 4 weeks.

The basal ration, modified slightly from that of Luckey et al. ('45), had the following composition: carbohydrate 61, hot alcohol extracted casein 18, gelatin 10, salts V (Briggs et al., '43) 6, soybean oil 4, feeding oil (2,000A–300D) 0.5, choline chloride 0.2, inositol 0.1, and L-cystine 0.3 gm per 100 gm of ration. The following were added in milligrams per kilogram: α-tocopherol 3.0, thiamine hydrochloride 3.0, niacin 50, calcium pantothenate 20, biotin 0.2, menadione 0.5, folic acid 4.0, *p*-aminobenzoic acid 100, riboflavin 6.0 and vitamin B_{12} 0.03. All of the rations were fed with and without 25 mg of aureomycin hydrochloride per kilogram of diet.

Pyridoxal, pyridoxamine or pyridoxine were added at equimolar levels as indicated in the tables. The rations were mixed at least every 10 days. Feed and water were supplied ad libitum.

RESULTS

With either glucose or autoclaved starch as the dietary carbohydrate, aureomycin was ineffective in stimulating growth at the low levels of pyridoxine (table 1). The marked sparing effect of aureomycin on the pyridoxine requirement

TABLE 1

Effect of aureomycin on the activity of pyridoxine with autoclaved starch or glucose as the dietary carbohydrate

LEVEL OF PYRIDOXINE · HCL PER GM OF DIET	AVERAGE WEIGHT OF CHICK AT 4 WEEKS [1]					
	Autoclaved starch				Glucose	
	Experiment 1		Experiment 2		Experiment 2	
	No aureomycin	Plus aureomycin	No aureomycin	Plus aureomycin	No aureomycin	Plus aureomycin
μg	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
0.25	66 [6]	74 [7]				
0.50	95 [3]	95 [1]	132 [6]	126 [6]	119 [4]	110 [2]
0.70	146	149 [3]	146 [1]	152 [3]	148	157
0.90	196	202	189	179 [1]	212 [1]	215 [1]
1.20	256	263	311	318	265	305 [1]

[1] Superscript indicates dead chicks. Twelve chicks started in experiment 1, 10 in experiment 2. Average day-old weight = 38 gm.

TABLE 2

Effect of feeding aureomycin with different carbohydrates and adequate vitamin B_6[1]

CARBOHYDRATE	VITAMIN B_6 ADDED		AVERAGE WEIGHT OF CHICK AT 4 WEEKS	
	Amount	Form	No antibiotic	Plus aureomycin
	μg/gm		*gm*	*gm*
Glucose	1.5	Pyridoxine · HCl	310	349
	2.0	Pyridoxine · HCl	330	341
Sucrose	4.0	Pyridoxine · HCl	266	309
	4.0	Pyridoxal · HCl	297	338
	4.7	Pyridoxamine · 2 HCl	246	288
Autoclaved starch	2.0	Pyridoxine · HCl	380	397
	2.0	Pyridoxal · HCl	378	396
	5.0	Pyridoxine · HCl	368	412
	5.0	Pyridoxal · HCl	356	387

[1] Experiments involving the different carbohydrates were not run concurrently. At least 10 chicks were used per group.

TABLE 3

Effect of carbohydrate source and aureomycin on the comparative activities of pyridoxal, pyridoxamine and pyridoxine

CARBOHYDRATE (%) AND VITAMIN B_6 (μG/GM) IN DIET	GROWTH AND SURVIVAL [1] AT 4 WEEKS			
	No aureomycin		Plus aureomycin	
	Av. wt.	Survival	Av. wt.	Survival
	gm		*gm*	
Glucose (61%)				
Pyridoxine · HCl (1.2)	231	40/46	270	44/46
Pyridoxal · HCl (1.2)	192	31/33	225	32/33
Pyridoxamine · 2 HCl (1.4)	180	25/33	212	28/33
Pyridoxine · HCl (0.70)	144	20/20	176	20/20
Pyridoxal · HCl (0.70)	137	16/20	150	15/20
Pyridoxamine · 2 HCl (0.82)	123	12/20	118	12/20
Glucose (41%) + lactose (20%)				
Pyridoxine · HCl (1.2)	288	19/20	332	19/20
Pyridoxal · HCl (1.2)	223	20/20	250	20/20
Pyridoxamine · 2 HCl (1.4)	177	16/20	212	16/20
Pyridoxine · HCl (0.70)	210	17/20	197	18/20
Pyridoxal · HCl (0.70)	170	11/20	174	11/20
Pyridoxamine · 2 HCl (0.82)	170	7/20	141	8/20
Sucrose (61%)				
Pyridoxine · HCl (1.2)	208	10/10	257	10/10
Pyridoxal · HCl (1.2)	129	8/10	141	9/10
Pyridoxamine · 2 HCl (1.4)	142	10/10	190	10/10
Sucrose (41%) + lactose (20%)				
Pyridoxine · HCl (1.2)	254	10/10	309	10/10
Pyridoxal · HCl (1.2)	223	9/10	199	9/10
Pyridoxamine · 2 HCl (1.4)	180	9/10	253	10/10
Autoclaved starch (61%)				
Pyridoxine · HCl (1.2)	305	30/30	316	29/30
Pyridoxal · HCl (1.2)	294	29/30	343	30/30
Pyridoxamine · 2 HCl (1.4)	284	29/30	335	30/30
Pyridoxine · HCl (0.70)	156	30/32	165	26/32
Pyridoxal · HCl (0.70)	143	26/32	154	26/32
Pyridoxamine · 2 HCl (0.82)	176	30/32	173	31/32
Autoclaved starch (41%) + lactose(20%)				
Pyridoxine · HCl (1.2)	301	10/10	344	10/10
Pyridoxal · HCl (1.2)	315	10/10	321	10/10
Pyridoxamine · 2 HCl (1.4)	307	10/10	343	10/10

[1] Survival is expressed as the number of chicks surviving the 4-week test period divided by the number of chicks started. No group contained less than 10 birds. Multiples of that number represent the average of several groups run at different times.

noted with the rat (Linkswiler et al., '51) is thus not present with rations of this type in the chick. At the adequate levels of vitamin B_6 shown in table 2, the diet becomes complete with respect to known vitamins, and the familiar growth-promoting properties of the antibiotic then become apparent.

The effects of aureomycin and of variation in the carbohydrate on the comparative activities of pyridoxal, pyridoxamine and pyridoxine are shown in table 3. In confirmation of previous results (Sarma et al., '46a) pyridoxal and pyridoxamine are less active than pyridoxine in rations high in soluble carbohydrate, whether this is glucose or sucrose. Some stimulation of growth by the antibiotic is evident, but it exerts little or no effect on the comparative activities of the three forms of vitamin B_6, in contrast to the result found in rats (Linkswiler et al., '51). With autoclaved starch, the result is notably different. No statistically significant differences in the activities of the three compounds are observed, either in the presence or absence of the antibiotic. The results using autoclaved starch as the dietary carbohydrate do not confirm the report of Luckey et al. ('45) that pyridoxal and pyridoxamine are less active than pyridoxine. This difference may be due to the fact that their ration contained no added source of vitamin B_{12}.

The effects of additions of 20% of lactose to diets suboptimal in vitamin B_6 are of considerable interest. Marked stimulation of growth resulted in those diets based on glucose or sucrose, but little or none resulted with autoclaved starch. The comparative activities of the three forms of vitamin B_6 were unchanged by this addition. Microbiological analysis of the lactose revealed that no vitamin B_6 was present.

DISCUSSION

Several conditions were cited in the introduction under which pyridoxal, pyridoxamine and pyridoxine showed equal activities in promoting growth of rats and chicks. The equal activities of the three compounds for chicks when diets based upon autoclaved starch are employed is another instance of

this behavior. It should be pointed out that this type of diet simulates natural diets much more closely than do those containing up to 61% of soluble carbohydrate. It is only on this latter type of diet that pyridoxal and pyridoxamine have proved less active than pyridoxine. Perhaps, therefore, this difference in activity does not exist under conditions of practical nutrition.

In explanation of the differences in activity observed on diets high in soluble carbohydrates, it was suggested (Sarma et al., '46a) that pyridoxal and pyridoxamine were utilized more readily than pyridoxine by the intestinal flora. When injected, or when fed apart from other food so that absorption was rapid, the three compounds should and do show equal activities. When mixed with the ration, absorption was considered as being delayed, so that bacteria, which either selectively destroy or utilize pyridoxal and pyridoxamine in preference to pyridoxine, compete with the host for these substances. One group of organisms for which this is demonstrably true are the lactic acid bacteria, which are among the most numerous in the gastrointestinal tract. They occur in substantial numbers in the duodenum and small intestine as well as in the colon (Shapiro and Sarles, '49; Shapiro et al., '49) and are greatly increased in the upper portion of the intestinal tract by feeding diets high in soluble carbohydrates (Johansson et al., '48). When diets based on starch are fed, the numbers of these organisms in the duodenum, but not in the lower portions of the tract, are decreased. The effects of various carbohydrates on the bacterial population of the upper portion of the intestinal tract, where the most active absorption of nutrients occurs, thus correlate with their effects upon the comparative activities of the forms of vitamin B_6, and lend support to the view that these differences result from a competition between bacterium and host for these essential nutrients.

The action of aureomycin in eliminating the observed differences in activity for the rat was interpreted (Linkswiler et al., '51) as indicating a relative decrease in population

of those organisms that compete with the host for vitamin B_6. According to the views developed above, this decrease need appear only in the numbers of microorganisms occurring in the upper portions of the intestinal tract, and not in the total count. Evidence for this selective decrease in numbers in the aureomycin-fed rat has recently appeared (Johansson et al., '52). Similar data for the chick are not available; however, the comparative activities of the three compounds in the chick are unchanged by the level of aureomycin used here. These differences in the rat and chick may reflect the recognized difference in composition of the intestinal flora of the two species (Evenson, '47), or other unidentified variables.

SUMMARY

1. Pyridoxal and pyridoxamine are less active than pyridoxine in supporting chick growth on otherwise complete rations containing large amounts (61%) of glucose or sucrose. The three compounds are equally active when autoclaved starch is the dietary carbohydrate. The latter type of ration simulates most closely those used in practical nutrition. The comparative activities of the three compounds on these rations is unchanged by feeding aureomycin at a level of 25 mg per kilogram of diet.

2. Substantial increases in growth with diets suboptimal in vitamin B_6 resulted when 20% of lactose was added to diets based on glucose or sucrose. No such increase resulted from addition of lactose to the starch-containing diet. The comparative activities of the three forms of vitamin B_6 are not changed by addition of this amount of lactose to any of these diets.

3. Possible explanations for the observed differences in activity of pyridoxal, pyridoxamine and pyridoxine with the composition of the ration are considered. The results are consistent with known changes in composition of the intestinal flora, and indicate that competition of intestinal flora with the host for limited supplies of these nutrients may explain

the differences in relative activities of these compounds that occur with differences in the diet or mode of administration.

LITERATURE CITED

BRIGGS, G. M., JR., T. D. LUCKEY, C. A. ELVEHJEM AND E. B. HART 1943 Studies on two chemically unidentified water-soluble vitamins necessary for the chick. J. Biol. Chem., *148:* 163–172.

DEBEY, H. J., E. E. SNELL AND C. A. BAUMANN 1952 Studies on the interrelationship between methionine and vitamin B_6. J. Nutrition, *46:* 203–214.

EVENSON, A. E. 1947 The intestinal flora of laboratory animals and its modification by diet and drugs. Unpublished Ph.D. Thesis. University of Wisconsin Library, Madison, Wisconsin.

JOHANSSON, K. R., G. E. PETERSON AND E. C. DICK 1952 The intestinal flora and vitamin B_{12} synthesis in rats fed aureomycin. Bact. Proc., p. 60.

JOHANSSON, K. R., W. B. SARLES AND S. K. SHAPIRO 1948 The intestinal microflora of hens as influenced by various carbohydrates in a biotin-deficient ration. J. Bact., *56:* 619–634.

LINKSWILER, H., C. A. BAUMANN AND E. E. SNELL 1951 Effect of aureomycin on the response of rats to various forms of vitamin B_6. J. Nutrition, *43:* 565–573.

LUCKEY, T. D., G. M. BRIGGS, JR., C. A. ELVEHJEM AND E. B. HART 1945 Activity of pyridoxine derivatives in chick nutrition. Proc. Soc. Exp. Biol. Med., *58:* 340–344.

MILLER, E. C., AND C. A. BAUMANN 1945 Further studies on factors that affect xanthurenic acid excretion. J. Biol. Chem., *159:* 173–183.

RABINOWITZ, J. C., AND E. E. SNELL 1948 The vitamin B_6 group. XIV. Distribution of pyridoxal, pyridoxamine, and pyridoxine in some natural products. Ibid., *176:* 1157–1167.

ROBBLEE, A. R., C. A. NICHOL, W. W. CRAVENS, C. A. ELVEHJEM AND J. G. HALPIN 1948 The effect of hen diet and chick diet on the need of chicks for an unidentified growth factor. Poultry Sci., *27:* 442–447.

SARMA, P. S., E. E. SNELL AND C. A. ELVEHJEM 1946a The vitamin B_6 group. VIII. Biological assay of pyridoxal, pyridoxamine, and pyridoxine. J. Biol. Chem., *165:* 55–63.

———— 1946b The vitamin B_6 group. IX. Comparative growth and antianemic potencies of pyridoxal, pyridoxamine and pyridoxine for dogs. Proc. Soc. Exp. Biol. Med., *63:* 284–286.

SHAPIRO, S. K., R. A. RHODES AND W. B. SARLES 1949 Lactobacilli in the intestinal tract of the chicken. J. Bact., *58:* 689–694.

SHAPIRO, S. K., AND W. B. SARLES 1949 Microorganisms in the intestinal tract of normal chickens. Ibid., *58:* 531–544.

SNELL, E. E., AND A. N. RANNEFELD 1945 The vitamin B_6 group. III. The vitamin activity of pyridoxal and pyridoxamine for various organisms. J. Biol. Chem., *157:* 475–489.

EFFECT OF TERRAMYCIN AND CERTAIN PHENYLARSONIC ACID DERIVATIVES ON THE GROWTH AND INTESTINAL FLORA OF TURKEY POULTS

G. W. ANDERSON, J. D. CUNNINGHAM AND S. J. SLINGER
Departments of Bacteriology and Poultry Husbandry, Ontario Agricultural College, Guelph, Canada

(Received for publication April 7, 1952)

Morehouse and Mayfield ('46) reported that 3-nitro-4-hydroxyphenyl arsonic acid had growth-stimulating properties for chickens and for turkeys. Bird et al. ('48) confirmed this observation in chickens and found that phenylarsonic acid and *p*-hydroxyphenyl arsonic acid were comparable in their activity to 3-nitro-4-hydroxyphenyl arsonic acid. *M*-nitrophenyl arsonic acid also showed some growth-promoting activity while *p*-chlorophenyl arsonic acid, sodium arsenate, *m*-nitrobenzene sulfonic acid, salicyclic acid, 3-nitrosalicylic acid and sodium *p*-phenolsulfonate were inactive. Morehouse ('49) found the optimum concentration of 3-nitro-4-hydroxyphenyl arsonic acid in the feed of chickens to be about 0.009%. The latter worker also noted that feed was utilized more efficiently by turkeys receiving 3-nitro-4-hydroxyphenyl arsonic acid than by their controls.

It has been amply demonstrated that certain antibiotics stimulate the growth of chicks and poults. Davis and Briggs ('51) have reviewed the early literature in this connection. These authors found that the chick and poult showed improved feed efficiency when the diet was supplemented with an antibiotic and stated that there was no indication that growth stimulation occurred as a simple manifestation of increased feed consumption. On the other hand, it had been

suggested earlier by the data of Scott and Glista ('50) that under their conditions aureomycin and 3-nitro-4-hydroxyphenyl arsonic acid stimulated growth in chicks through the medium of increased feed consumption.

Many workers have shown that diet influences the intestinal microflora of chickens. Johansson et al. ('48) observed a change in the fecal microflora associated with dietary carbohydrate in pullets. The bacterial types reported were coliforms, enterococci and lactobacilli. Their data also suggested that anaerobic lactobacilli exist in the intestines of chickens in considerable numbers. Moore et al. ('46) found that orally administered streptomycin brought about a reduction in coliform bacteria in the cecal contents of chicks. Elam et al. ('51) reported that feeding penicillin caused a significant increase in total numbers of intestinal microorganisms as indicated by thioglycollate counts. This antibiotic also caused a significant increase in enterococci. The administration of penicillin either orally or intravenously resulted in a marked increase in penicillin resistant organisms in the intestinal tract. Anderson et al. ('52) reported that dietary penicillin effected a reduction in the pH of the cecal contents, which was associated with an increase in numbers of lactobacilli, aciduric, anaerobic and microaerophilic, aerobic and coliform types of organisms. The increase in coliforms was due to the presence of an atypical strain of *E. coli.* This strain lacked the characteristic green metallic sheen, fermented lactose slowly and did not attack dulcitol. Enterococci counts were reduced by the addition of penicillin to the diet.

The present experiment was made to determine the influence of terramycin and certain phenylarsonic acid derivatives on the growth and cecal flora of turkey poults.

MATERIALS AND METHODS

Day-old Broad Breasted Bronze female poults were weighed individually and distributed into experimental groups on the basis of weight. Each group was comprised of 18 poults.

The birds were reared in electrically heated battery brooders with raised wire floors.

The percentage composition of the basal diet was as follows: corn 16, wheat 19, oat groats 5, dehydrated alfalfa 2, soybean oilmeal (44% protein) 50, limestone 1.5, steamed bone meal 5.0, iodized salt 0.5, fish oil (2,400 A and 400 D per gram) 1.0. In addition, to each 100 lb. of diet the following were added: manganese sulfate (technical) 7 gm, riboflavin 0.2 gm, niacin 0.5 gm, and vitamin B_{12} 0.1 mg. When included, terramycin hydrochloride was present to the extent of 15 mg per kilogram, and the various phenylarsonic acid derivatives in the amount of 70 mg per kilogram of diet. The experimental diets and water were supplied ad libitum.

The birds were weighed individually at 21 and 30 days of age and the feed consumption was recorded. Two birds of average weight were removed from each group after every weighing and sacrificed for bacteriological analysis of the cecal contents. They were aseptically opened and the ceca ligatured off. The outer surface was then washed with a mercuric chloride solution and removed to sterile petri dishes. The cecal contents were expressed by the use of a sterile spatula and forceps into a second series of sterile petri dishes.

The pH of the various samples was recorded using a Beckman pH meter and employing contact electrodes applied to the thoroughly mixed samples. A 1-gm sample of the cecal contents was added to a 99-ml sterile distilled H_2O blank containing glass beads. The sample was thoroughly shaken on an automatic shaking machine for 5 minutes. From this initial dilution, dilutions of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} were made and duplicate 1-ml samples of each transferred to the media indicated below.

Levine's eosin methylene blue agar was employed for the enumeration and differentiation of the members of the coliform group.

"S. F." broth of Hajna and Perry ('43) dispersed in 10 ml amounts in test tubes was used for the cultivation of en-

terococci. The M. P. N. method was used for the enumeration of this group of microorganisms.

Tomato juice agar (Difco) was chosen for plating the samples for the isolation and counting of lactobacilli. The plates were overlaid with 2% agar to reduce O_2 tension.

Tryptone glucose extract agar with 2% yeast extract (Difco) was used for the cultivation and enumeration of aerobes. This same medium with 1 ml per liter of a 1.6% alcoholic solution of Brom Cresol Purple and 2% skim milk was used for the cultivation and enumeration of the aciduric and proteolytic types of organisms.

Linden's thyoglycollate medium (Difco) was placed in test tubes in 10 ml amounts for the cultivation of anaerobes. The M. P. N. method was used for counting these organisms. A 2% agar solution was added after inoculating the tubes; this formed a plug and helped prevent reabsorption of O_2. The above media were incubated at 37°C. for 48 and 72 hours with the exception of the "S. F." medium which was incubated at 41°C. for 72 hours. Viable bacterial counts were recorded following incubation. From the plates representing the highest dilutions which supported the growth of a minimum of 30 colonies, representative colonies were "fished" off and maintained on either tryptone glucose yeast extract agar or tomato juice agar slants as stock cultures. A total of 225 cultures was selected on the basis of their colony characteristics. These cultures were examined as soon as possible after isolation, with a minimum of transferring in order to prevent form variation. Cultures were studied for colony characteristics and Gram reaction. Any mixed cultures observed were restreaked and isolated colonies picked off in order to obtain pure cultures.

RESULTS

Observations of growth

The weight and feed efficiency data are presented in table 1. The final weight data were examined statistically by the

TABLE 1

Effect of terramycin and certain phenylarsonic acid derivatives on the growth of turkey poults

GROUP NO.	PHENYLARSONIC ACID DERIVATIVE	TERRAMYCIN HCl	AGE — 21 DAYS		AGE — 30 DAYS		TOTAL FEED CONSUMED
			Weight	Feed/Gain	Weight	Feed/Gain	
			gm.		*gm.*		*lb.*
1	None	—	271 (18)[1]	2.00	494 (16)[1]	2.08	34.25
2	None	+	341 (18)	1.91	585 (16)[2]	2.05	41.00
3	Magnesium 4-hydroxyphenylarsonate	—	310 (18)	1.90	550 (16)[2]	2.04	38.00
4	Magnesium 4-hydroxyphenylarsonate	+	325 (18)	1.84	568 (16)[2]	2.02	39.00
5	3-amino 4-hydroxyphenyl arsonic acid	—	287 (18)	2.00	522 (16)	2.12	37.25
6	3-amino 4-hydroxyphenyl arsonic acid	+	330 (17)	1.93	596 (15)[2]	2.13	40.50
7	3-acetylamino 4-hydroxyphenyl arsonic acid	—	276 (18)	1.99	506 (16)	2.20	37.25
8	3-acetylamino 4-hydroxyphenyl arsonic acid	+	343 (18)	1.88	617 (16)[2]	1.88	39.75
9	3-nitro 4-hydroxyphenyl arsonic acid	—	312 (18)	1.85	560 (16)[2]	1.99	37.75
10	3-nitro 4-hydroxyphenyl arsonic acid	+	335 (18)	1.82	590 (16)[2]	1.98	39.75

[1] Figures in parentheses indicate number of birds surviving.

[2] Weight significantly greater than group 1 ($P < 0.01$).

method of analysis of variance. An "F" value was obtained indicating highly significant differences due to diets. Comparing the mean square with the error mean square gave a measure of the significance of the differences between means. It will be noted that additions to the basal diet of terramycin alone or in combination with any of the phenylarsonic acid derivatives resulted in weight increases which were highly significant at 30 days of age. In the absence of terramycin highly significant weight increases resulted from the inclusion of magnesium 4-hydroxyphenylarsonate and 3-nitro-4-hydroxyphenyl arsonic acid. The weight increases caused by the 3-amino- and 3-acetylamino-derivatives were not significant at $P = 0.05$. In general, significant increases in weight were accompanied by slight improvement in feed efficiency.

The weight increases caused by adding terramycin to diets containing magnesium 4-hydroxyphenylarsonate or the 3-nitro-derivative were not significant. On the other hand, considered on the basis of both weight and feed efficiency there appears to be a slight advantage in favor of combining either of these compounds with the antibiotic at the levels employed. In addition, while the mean weight of the group fed the 3-acetylamino-derivative in the presence of terramycin was not significantly greater than that of the group fed antibiotic alone, when feed efficiency is also considered, there appears to be a synergistic relationship between terramycin and the acetylamino-derivative.

On the basis of the growth-promoting activity in chicks of phenylarsonic acid and its nitro- and hydroxy-derivatives, Bird at al. ('48) concluded that the presence of the nitro and hydroxy groups was not essential. In the present experiment the 3-nitro-derivative both in the absence and the presence of terramycin, resulted in slightly (but not significantly) greater weight and somewhat better feed efficiency than the magnesium 4-hydroxyphenylarsonate. This suggests that, at the levels used, the latter compound was somewhat inferior to the former. The lack of significant response to the 3-amino- and 3-acetylamino-derivatives suggests

that the presence of these substances reduced the effectiveness of the compound in the absence of antibiotic.

Bacteriological findings

The results of the pH measurements and viable cecal floral counts are presented in tables 2 and 3. Each value represents the average of determinations made on two birds. It will be noted that, at both 21 and 30 days of age, terramycin either alone or in combination with the various phenylarsonic acid derivatives effected a lowering of the pH of the cecal contents. In the absence of antibiotic the phenylarsonic acid derivatives, with one exception, caused a decrease in the pH of the cecal contents. The addition of terramycin in the presence of the various phenylarsonic acid derivatives, again with a single exception, resulted in a lower pH than with the phenylarsonic acid derivative alone.

In most cases, aerobic counts were reduced by additions to the basal diet of terramycin, phenylarsonic acid derivatives or combinations thereof. However, the 3-amino-derivative, and the 3-nitro-derivative in the presence of terramycin, caused an increase in aerobes. This group of organisms was made up of the following types: (a) Gram-negative short rods occurring singly, facultative and motile; (b) Gram-negative coccoid forms to long rods — single or in pairs, non-motile, non-spore-forming and facultative; (c) Gram-negative long rods — single, non-motile, non-spore-forming and facultative. Variation in numbers of aerobes due to diet was caused mainly by differences in organisms belonging to (b).

Anaerobic counts were higher than aerobic counts. Terramycin, phenylarsonic acid derivatives and combinations of these materials caused a reduction in anaerobic counts at 21 days of age. At 30 days of age, the influence of the dietary supplements on the counts of these organisms was variable. The following types of organisms were observed in this group: (a) Gram-positive cocci in chains and masses — non-motile, non-spore-forming and microaerophilic; (b) Gram-negative short, fat rods with rounded ends — single and in pairs, non-

TABLE 2

Effect of terramycin and certain phenylarsonic acid derivatives on the cecal microflora of 21-day-old poults
(Counts indicate numbers of organisms per gram of cecal contents)

GROUP NO.	PHENYLARSONIC ACID DERIVATIVE	pH CECAL CONTENTS	TERRAMYCIN HCl	AEROBES ($\times 10^8$)	ANAEROBES ($\times 10^8$)	ACIDURIC ($\times 10^8$)	PROTEOLYTIC ($\times 10^6$)	LACTOBACILLI ($\times 10^6$)	COLIFORMS ($\times 10^5$)	ENTEROCOCCI ($\times 10^4$)
1	None	6.20	—	30.0	12.0	2.1	91.0	5.0	24.0	11.0
2	None	5.65	+	1.7	1.8	1.2	5.0	22.0	13.2	10.0
3	Magnesium 4-hydroxy-phenylarsonate	5.85	—	1.0	0.2	1.0	0.3	2.0	31.4	13.0
4	Magnesium 4-hydroxy-phenylarsonate	5.75	+	1.6	<0.0	1.1	5.0	220.0	10.3	11.0
5	3-amino 4-hydroxy-phenyl arsonic acid	5.80	—	38.0	1.7	34.0	40.0	30.0	28.0	14.0
6	3-amino 4-hydroxy-phenyl arsonic acid	5.50	+	1.2	0.5	0.6	6.0	5.0	5.2	100.0
7	3-acetylamino 4-hydroxy-phenyl arsonic acid	6.35	—	2.0	0.2	1.0	10.0	16.0	13.2	150.0
8	3-acetylamino 4-hydroxy-phenyl arsonic acid	5.80	+	15.0	<0.0	12.0	30.0	16.0	12.0	149.0
9	3-nitro 4-hydroxy-phenyl arsonic acid	5.75	—	1.1	1.4	1.5	0.5	10.0	11.0	10.0
10	3-nitro 4-hydroxy-phenyl arsonic acid	5.95	+	111.0	1.1	107.0	40.0	220.0	35.0	132.0

TABLE 3

Effect of terramycin and certain phenylarsonic acid derivatives on the cecal microflora of 30-day-old poults
(Counts indicate numbers of organisms per gram of cecal contents)

GROUP NO.	PHENYLARSONIC ACID DERIVATIVE	pH CECAL CONTENTS	TERRAMYCIN HCl	AEROBES ($\times 10^8$)	ANAEROBES ($\times 10^7$)	ACIDURIC ($\times 10^6$)	PROTEOLYTIC ($\times 10^5$)	LACTOBACILLI ($\times 10^6$)	COLIFORMS ($\times 10^5$)	ENTEROCOCCI ($\times 10^4$)
1	None	5.95	—	7.3	0.1	7.2	1.0	9.0	0.5	1.0
2	None	5.70	+	3.5	0.9	3.3	2.0	34.0	0.1	11.0
3	Magnesium 4-hydroxyphenylarsonate	5.65	—	2.6	< 0.0	2.6	0.4	12.0	0.7	13.1
4	Magnesium 4-hydroxyphenylarsonate	5.40	+	1.9	10.0	12.0	7.2	101.0	30.0	500.0
5	3-amino 4-hydroxyphenyl arsonic acid	5.65	—	12.0	2.0	10.0	20.0	5.0	247.0	25.0
6	3-amino 4-hydroxyphenyl arsonic acid	5.50	+	5.5	0.7	5.3	2.0	14.0	2.6	19.0
7	3-acetylamino 4-hydroxyphenyl arsonic acid	5.85	—	3.9	< 0.0	3.9	0.2	0.7	0.1	31.0
8	3-acetylamino 4-hydroxyphenyl arsonic acid	5.60	+	0.4	< 0.0	0.4	0.3	160.0	8.9	29.1
9	3-nitro 4-hydroxyphenyl arsonic acid	5.50	—	4.3	10.0	4.1	2.0	39.0	54.0	0.1
10	3-nitro 4-hydroxyphenyl arsonic acid	5.50	+	11.5	0.1	11.1	4.0	16.0	31.0	1.9

spore-forming, non-motile and microaerophilic. The numbers of the different types did not appear to be consistently related to the diet.

The aciduric types of organisms were increased with certain supplements and decreased with others. This group comprised Gram-negative cocci, short rods and long rods. These were non-spore-forming and aerobic. Proteolytic organisms were much less numerous at 30 days of age than at 21 days of age. At 21 days all dietary treatments caused a reduction in proteolytic counts while at 30 days of age this trend was by no means consistent. The proteolytic types of organisms were Gram-negative cocci and short rods, occurring singly. All these organisms were non-motile and facultative with the rods being spore-formers.

The lactobacilli counts at 21 days of age were increased, in most cases, by the addition of terramycin, phenylarsonic acid derivatives or combinations of these compounds. However, in the case of the diet containing magnesium 4-hydroxyphenylarsonate in the absence of antibiotic, and that containing the 3-amino-derivative in the presence of antibiotic, these counts were not increased. It will be noted that, at 30 days of age, only in the case of the 3-amino-derivative and the 3-acetylamino-derivative, in the absence of terramycin, were the lactobacilli counts not increased as compared to the basal diet. The lactobacilli isolated from tomato juice agar appeared as pin-point colonies and grew below the surface. The cells were Gram-positive rods occurring singly, in pairs or as long chains. They exhibited varying degrees of pleomorphism and displayed granulation when Gram-stained. The age and amount of culturing influenced the morphology of the cells. The cells were non-motile and microaerophilic. Pleomorphism was more marked in the cells from birds receiving terramycin or the phenylarsonic acid derivatives, or both, than in cells isolated from birds fed the basal diet. Considerable difficulty was experienced in maintaining the strains which showed variation.

The data concerning coliform counts indicate that these organisms were influenced by dietary treatments. The effect was greater at 30 days than at 21 days of age. In some instances the treatment tended to decrease the coliform counts and in other cases there appeared to be marked increases in the numbers of these organisms as a result of the supplements used. The coliforms were Gram-negative short rods or coccoid to long rods. They occurred singly, were non-spore-forming, motile or non-motile and facultative. In the presence of either terramycin or phenylarsonic acid derivatives, or both, the coliforms differed from those found in birds fed the basal diet in that many of the former failed to give a normal Imvic's reaction, fermented lactose slowly, did not attack dulcitol and failed to give the same characteristic green metallic sheen.

At 21 days of age certain dietary treatments caused an increase in enterococci organisms while others appeared to bring about little or no change. At 30 days of age the enterococci were increased by all diets excepting those containing the 3-nitro-derivative. This group of organisms consisted of Gram-positive coccoid forms. The cells were spherical in shape and occurred in pairs of short chains. They were non-motile, facultative anaerobes.

DISCUSSION

A point of interest in this work is the relatively similar influence of terramycin and certain phenylarsonic acid derivatives on the cecal floral counts and on the pH of the cecal contents. It is also noteworthy that increases in weight were accompanied in most cases by a reduction in pH of the cecal contents. The reduction in pH caused by the 3-acetylamino-derivative in the absence of antibiotic was less than that resulting from supplementation with the other phenylarsonic acid derivatives and the former compound failed to produce a significant growth response. In addition, the decreases in pH resulting from the inclusion of terramycin in diets containing the various phenylarsonic acid derivatives

were accompanied by weight increases — albeit not significant in all cases. These results suggest that the reduction in pH of the cecal contents may be associated with the growth stimulation brought about by terramycin and phenylarsonic acid derivatives.

While most dietary treatments caused a reduction in aerobes accompanied by an increase in growth, birds fed the diet containing the 3-nitro-derivative and terramycin showed an increase in these organisms, and a significant increase in growth. Furthermore, the 3-acetylamino-derivative in the absence of antibiotic caused a marked reduction in aerobes and no significant increase in growth. These results suggest that changes in aerobic counts are not necessarily correlated with growth.

The decrease in numbers of anaerobes at 21 days of age, caused by dietary treatment, did not appear to be closely associated with changes in growth. Similarly, the counts of these organisms varied widely at 30 days of age and appeared to bear little relationship to growth changes. It would also appear that changes in numbers of aciduric, proteolytic, coliform and enterococci organisms were not consistently involved in the growth-promoting effect of terramycin and phenylarsonic acid derivatives.

With respect to lactobacilli, at 30 days of age only in the case of the diets containing the 3-amino-derivative and the 3-acetylamino-derivative, in the absence of antibiotic, were the counts of this group of organisms not increased as compared to the basal diet. Furthermore, these were the only dietary treatments which did not result in a significant improvement in weight. Another interesting point is that the diet containing the 3-acetylamino-derivative in combination with terramycin resulted in the highest lactobacilli count and the greatest numerical weight. While there were two exceptions to an increase in weight being accompanied by a greater number of lactobacilli organisms at 21 days of age, the data as a whole suggest that the weight increases caused by ter-

ramycin and phenylarsonic acid derivatives were associated with increased counts of lactobacilli.

SUMMARY

Highly significant increases in the weight of female turkey poults were obtained by inclusion in the diet of magnesium 4-hydroxyphenylarsonate and 3-nitro-4-hydroxyphenyl arsonic acid.

The weight increases caused by including 3-amino-4-hydroxyphenyl arsonic acid and 3-acetylamino-4-hydroxyphenyl arsonic acid were not significant.

Additions to the basal diet of terramycin alone or in combination with any of the phenylarsonic acid derivatives resulted in highly significant increases in weight. For the most part, weight increases were accompanied by a slight improvement in feed efficiency.

The increases in weight caused by terramycin, phenylarsonic acid derivatives or combinations thereof, were accompanied in most cases by a reduction in the pH of the cecal contents.

Terramycin, phenylarsonic acid derivatives and combinations of these materials markedly influenced the cecal flora. The effects of the antibiotic and some of the phenylarsonic acid derivatives were similar in many respects.

In general, weight increases were accompanied by increases in the lactobacilli counts of the cecal contents. The numbers of aciduric, proteolytic, coliform and enterococci organisms were not consistently in agreement with increases in weight.

ACKNOWLEDGMENT

We wish to thank the Synthetic Drug Co., Ltd., Toronto, Canada, for the phenylarsonic acid derivatives; Charles Pfizer and Co., Inc., Brooklyn, New York., for the terramycin hydrochloride; and Merck and Co., Ltd., Montreal, Canada, for the niacin and vitamin B_{12} used in this work.

LITERATURE CITED

ANDERSON, G. W., J. D. CUNNINGHAM AND S. J. SLINGER 1952 Effect of protein level and penicillin on growth and intestinal flora of chickens. J. Nutrition, *47:* 175–189.

BIRD, H. R., A. C. GROSCHKE AND MAX RUBIN 1948 Effects of arsonic acid derivatives in stimulating growth of chickens. Ibid., *37:* 215–226.

DAVIS, R. L., AND G. M. BRIGGS 1951 Studies with antibiotics in chick and poult starting rations. Poultry Sci., *30:* 767–771.

ELAM, J. F., L. L. GEE AND J. R. COUCH 1951 The effect of feeding penicillin on the life cycle of the chick. Proc. Soc. Exp. Biol. Med., *77:* 209–213.

HAJNA, A. A., AND C. A. PERRY 1943 Comparative study of presumptive and confirmative media for bacteria of the coliform group and fecal streptococci. Am. J. Pub. Health, *33:* 550–556.

JOHANSSON, K. R., W. B. SARLES AND S. K. SHAPIRO 1948 The intestinal microflora of hens as influenced by various carbohydrates in a biotin-deficient ration. J. Bact., *56:* 619–634.

MOORE, P. R., A. EVENSON, T. D. LUCKEY, E. MCCOY, C. A. ELVEHJEM AND E. B. HART 1946 Use of sulfasuxidine, streptothricin and streptomycin in nutritional studies with the chick. J. Biol. Chem., *165:* 437–441.

MOREHOUSE, N. F. 1949 Accelerated growth in chickens and turkeys produced by 3-nitro-4-hydroxyphenylarsonic acid. Poultry Sci., *28:* 375–384.

MOREHOUSE, N. F., AND O. T. MAYFIELD 1946 The effect of some aryl arsonic acids on experimental coccidiosis infection in chickens. J. Parasitol., *32:* 20–24.

SCOTT, H. M., AND W. R. GLISTA 1950 The effect of aureomycin and arsonic acid on chick growth. Poultry Sci., *29:* 921–923.

THE RELATION OF THE RATE OF GROWTH TO THE DIET. A STUDY OF A STOCK COLONY RATION FOR ALBINO RATS

REBECCA B. HUBBELL AND W. A. KREHL

The Connecticut Agricultural Experiment Station, and Yale Nutrition Laboratory, Department of Physiological Chemistry, Yale University, New Haven

(Received for publication July 8, 1952)

INTRODUCTION

In the management of an animal colony, the selection of the breeding stock and of the diet are important. As is well known, a colony of albino rats has been maintained at The Connecticut Agricultural Experiment Station in New Haven since 1910. In that year Osborne and Mendel obtained animals from a local pet shop and two years later a few extracted albinos were bought from Dr. Donaldson at the Wistar Institute. This original colony supplied rats for a similar one which was maintained for many years in the Department of Physiological Chemistry at Yale and during that period the strain became known as the "Yale Strain."

Inevitably, during the 40 years the colony has been in existence, there have been a few changes in the type of ration used. Mendel and Hubbell ('35) recorded the rate of growth and breeding performance of the animals at 4 intervals during the first 25 years and showed that an enhanced growth rate, which was first noted in 1931, could be attributed to the type of food supplied and not to the selection of unusually large animals for breeding. Similar data for each of the 15 years since that report was published show that there has been a marked uniformity in the weights of the young at weaning and that the average weaning weights for both males and females for the entire period are identical with

the 1935 values. However, there has been a decline in the percentage of litters born and weaned. For the year 1935, 93% of the matings were fertile and 90% of the litters were weaned. In 1951 the corresponding values were 79 and 84%, respectively. In addition to these changes, performance has been rather unsatisfactory in some other respects. A few mothers kill their young, many males are infertile, and respiratory disorders persist. In general it has been observed that these departures from normal are transitory and may possibly be attributable to variations in the composition of the natural foods employed.

Because these fluctuations as well as the general downward trend observed in recent years might indicate the need for a modification of the stock ration, a small scale breeding experiment has been carried through the F_4 generation. Rats fed the colony ration of "Calf Meal Food" (Mendel and Hubbell, '35) have been compared with those in two other groups in which this diet was supplemented in one case with 3% whole liver substance [1] and in the other with 5 ml of orange juice [2] per 50 gm of rat per day. Liver substance was selected because it supplies vitamin B_{12} and perhaps others factors not yet identified, and in addition furnishes valuable animal protein. In connection with studies carried out on the vitamin content of orange juice (Krehl and Cowgill, '50), and because of the possible role of ascorbic acid in the nutrition of the rat under certain circumstances (Mayer and Krehl, '48), it seemed of interest to test the effect of the addition of this product as a supplement to the diet.

EXPERIMENTAL

To initiate the experiment, 18 female and 6 male weanling rats were assigned to each of three groups started, for laboratory convenience, at monthly intervals. Each group was subdivided into three groups of 6 female and two male rats

[1] Whole liver substance, Wilson and Co., Chicago, Ill.

[2] Reconstituted from the frozen juice, purchased from a wholesale distributor as a uniform lot.

on the basis of the food to be tested. Between the ages of 110 and 120 days they were mated according to the regular procedure in this laboratory, namely three females and one male in each cage. At the end of three weeks the females were transferred to separate cages. All litters were reduced at birth to 8 animals and the young were weaned at 21 days of age. If no litter was born, the female was remated with a different male. There were no further matings of those females that cast young but failed to wean them.

RESULTS AND DISCUSSION

There was somewhat more rapid growth of male rats fed the liver supplement in comparison with the controls or those that received the orange juice. This was true in all generations and there were no consistent changes from one generation to another. Male rats in the liver groups attained an average weight of 421 ± 20 [3] gm at 110 days of age in contrast to 376 ± 31 gm for the controls and 350 ± 44 gm for those in the orange juice groups. The average weights of the females were essentially the same in all three groups, namely 251 ± 13 gm, 247 ± 14 gm, and 239 ± 18 gm, respectively, at 110 days. It is of interest to note the high variability in the weights of the males of the orange juice groups.

The reproduction data for the entire experiment are summarized in table 1. With respect to the percentage of litters born and weaned and the number of young born and weaned in each litter, the addition of liver substance or of orange juice had no effect. However, the average weaning weights of the rats in the orange juice group were markedly lower than those of the other two groups. For weanling females the *highest* value in the orange group was 43.8 ± 2.6 gm in the F_4 generation while the *lowest* in either of the other two groups was 46.7 ± 3.2 gm in the F_0 generation of those fed corresponding values were 45.9 ± 3.3 in the F_2 generation the diet supplemented with liver substance. For males the

[3] Standard deviation.

in contrast to 47.9 ± 3.8 in the F_0 generation of the control group. No record was kept of food intake so that it is impossible to determine whether these differences in weaning weights can be associated with the consumption of rather large amounts of orange juice and a resultant lowered food intake for the animals of this group. The orange juice was

TABLE 1

The effect on reproduction of the addition of liver substance or orange juice to a stock ration for rats

DIET	GENERATION	MATINGS [1]	LITTERS BORN	YOUNG BORN PER LITTER	LITTERS WEANED	YOUNG WEANED PER LITTER	AVERAGE WEANING WEIGHTS ♀	AVERAGE WEANING WEIGHTS ♂
			%		%		gm	gm
Control	F_0	21	76.2	11.8	68.8	6.6	47.8 ± 4.7	47.9 ± 3.8
	F_1	21	71.4	8.2	73.3	6.4	48.5 ± 4.2	50.0 ± 2.9
	F_2	19	78.9	8.4	86.7	6.2	50.0 ± 3.9	54.0 ± 5.1
	F_3	24	66.7	8.8	75.0	6.3	49.1 ± 4.0	50.7 ± 4.2
	F_4	19	84.2	8.9	68.8	7.0	48.9 ± 4.0	50.9 ± 4.6
Control + liver	F_0	21	76.2	10.5	81.3	7.5	46.7 ± 3.2	50.4 ± 1.4
	F_1	20	85.0	9.2	82.4	7.1	48.0 ± 4.5	50.5 ± 3.1
	F_2	21	81.0	6.8	58.8	6.5	48.9 ± 2.3	49.2 ± 1.8
	F_3	20	75.0	8.4	86.7	6.8	48.5 ± 3.8	51.4 ± 3.7
	F_4	25	52.0	7.8	61.5	7.3	48.3 ± 2.1	50.3 ± 2.0
Control + orange juice	F_0	24	66.7	10.1	87.5	6.6	43.2 ± 2.6	44.5 ± 4.2
	F_1	22	81.8	8.8	66.7	6.7	42.2 ± 4.7	43.6 ± 3.7
	F_2	20	80.0	9.6	62.5	7.1	42.9 ± 4.0	45.9 ± 3.3
	F_3	22	72.7	9.9	75.0	7.0	42.3 ± 6.3	45.6 ± 4.0
	F_4	23	73.9	8.4	76.5	6.3	43.8 ± 2.6	43.0 ± 2.8

[1] Matings in excess of 18 (6 for each group) indicate rematings that were necessary to obtain a litter.

supplied during the day and water and food were withheld until the juice had been consumed. In a very few cases the rats failed to drink the entire portion of juice.

It is interesting to compare the data from this brief experiment with the records of the entire colony for the past 15 years. The average percentage of litters born and weaned is definitely lower in all of the experimental groups than it is in the main colony. In the experimental groups about

75% of the females produced young and slightly less than 75% of the litters were weaned. This is in contrast to 83 and 87% for the larger group and is also below the record for the most recent single year, 1951. The poorer results in the experimental groups may be associated with the fact that animals for new generations were always selected from first litters, whereas in the main colony the progeny of second and third matings are used almost entirely and only occasional use is made of young from first matings. However, although fewer young were born and weaned than in the main colony, the weaning weights of the controls and of the liver-fed rats were slightly above the 15-year average of 47.1 gm for females and 48.5 gm for males.

SUMMARY

In an attempt to effect an improvement in the breeding ration for albino rats the standard stock ration for the colony, "Calf Meal Food," was supplemented with 3% whole liver substance or with 5 ml of orange juice for each 50 gm of rat per day. Breeding data were secured through the F_4 generation. In general it was found that in the group fed the orange juice supplement there was a decreased rate of growth during the lactation period, due possibly to a lowered food intake. The weaning weights of 21-day-old rats were markedly below those of the other two groups. The post-weaning growth of male rats fed the liver-supplemented food was somewhat more rapid than it was in either of the other two groups. There was no difference between the groups with respect to the percentage of fertile matings or of litters weaned, but in all groups these results were less satisfactory than in the main colony during the same period of time.

ACKNOWLEDGMENT

The authors are indebted to George R. Smith for technical assistance.

LITERATURE CITED

KREHL, W. A., AND G. R. COWGILL 1950 Vitamin content of citrus products. Food Research, *15:* 179–191.

MAYER, J., AND W. A. KREHL 1948 The relation of diet composition and vitamin C to vitamin A deficiency. J. Nutrition, *35:* 523–538.

MENDEL, L. B., AND R. B. HUBBELL 1935 The relation of growth to diet. III. A comparison of stock rations used in the breeding colony at The Connecticut Agricultural Experiment Station. Ibid., *10:* 557–563.

BORDEN AWARD IN NUTRITION

Nominations are solicited f· r the 1953 Award and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the Jury of Award may recommend that it will be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor

The formal presentation will be made at the annual meeting of the Institute in the spring of 1953. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 1, 1953. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

Chairman, Nominating Committee:

DR. LEO T. SAMUELS
Department of Biological Chemistry
University of Utah Medical School
Salt Lake City, Utah

OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of $1000.00 established by the Nutrition Foundation, Inc., for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. Nominations for the 1953 Award, accompanied by data relative to the accomplishments of the nominee, must be sent to the Chairman of the Nominating Committee before January 1, 1953.

Chairman, Nominating Committee:

DR. PHILIP HANDLER
Department of Biochemistry and Nutrition
Duke University School of Medicine
Durham, North Carolina

INDEX

ABNORMALITIES, production in young of multiple congenital, by maternal pteroylglutamic acid deficiency during gestation, 61.
ABRAHAM, J. See Curtin, L. V., 499.
Acrodynia, factors affecting development in pyridoxine-deficient rats, 325.
Adult rats (see Rats, adult).
Albino rat(s) (see Rat(s), albino).
ALEXANDER, J. C., AND D. C. HILL. The effect of heat on the lysine and methionine in sunflower seed oil meal, 149.
AMERICAN INSTITUTE OF NUTRITION, 115.
Editor of Journal of Nutrition, report, 116.
Election of officers for 1952–53, 119.
Committees for 1952–53, 122.
New members elected, 116.
Amino acid(s) and peptone ingestion, effect upon concentrations of tissue amino acids, 139.
—— availability to microorganisms, nutritional evaluation of food proteins by measuring. I., 231.
—— (tissue), effect of peptone and amino acid ingestion upon concentrations of, 139.
ANDERSON, G. W., J. D. CUNNINGHAM AND S. J. SLINGER. Effect of terramycin and certain phenylarsonic acid derivatives on the growth and intestinal flora of turkey poults, 539.
Antibiotics, influence on protein and energy utilization in low protein diet, 477.
Arginine, nitrogen balance of adult rats fed diets devoid of, 391.
ARNOLD, A., AND J. S. SCHAD. Evaluation of nitrogen source materials by an intraperitoneal rat test, 377.
Ascorbic acid, blood levels of, during ascorbic acid depletion and repletion, 49.
—— depletion and repletion, tyrosine metabolism and blood levels of ascorbic acid during, 49.
—— nutritional studies of the chinchilla, with special reference to, 31.
—— nutriture in the human. I. Tyrosine metabolism and blood levels of ascorbic acid during ascorbic acid depletion and repletion, 49.
ASLING, C. W. See Nelson, M. M., 61.
Award, Borden, in nutrition, announcement, 1953, 123, 296; presentation, 1952, 121.
— Osborne and Mendel, announcement, 1953, 124, 295; presentation, 1952, 121.
— Mead Johnson vitamin B-complex, presentation, 1952, 121.

B vitamin(s) deficiencies, lipogenesis in certain, 345.
—— synthesis of certain, in cobalt-deficient sheep, with special reference to vitamin B_{12}, 431.
Balance, nitrogen, of adult rats fed diets low in L- and DL-lysine, or devoid of arginine, 391.
Basal heat production and elimination of 13 normal women at temperatures from 22°C. to 35°C., 257.
— metabolism (see Metabolism, basal).
BEADLES, J. R. See Mitchell, H. H., 461.
BEARE, J. L. See Beaton, J. R., 325.
BEATON, J. R., J. L. BEARE AND E. W. MCHENRY. Factors affecting the development of acrodynia in pyridoxine-deficient rats, 325.
BECK, L. W. See Mattson, F. H., 335.
BENEDICT, J. H. See Mattson, F. H., 335.
BERGEIM, O. See Sheffner, A. L., 139.
BERGQUIST, L. M. See Cheng, A. L. S., 161.
Betaine, effect of vitamin B_{12} upon utilization by young poult, 201.
Biological values of proteins in fish meals, evaluation by nitrogen retention method, 401.
BLACK, A. See Knoebel, L. K., 477.
Blood levels of ascorbic acid during ascorbic acid depletion and repletion, 49.
— of swine fed supplemental levels of pantothenic acid, pantothenic acid content of, 317.
— thiamine values, daily. Thiamine metabolism of women on controlled diets. II., 307.
BLUM, A. E. See Horn, M. J., 231.
BOOTH, V. H. Liver storage of vitamin A by male and female rats, 13.
Borden award in nutrition, announcement, 1953, 123, 296; presentation, 1952, 121.
BOWLAND, J. P. See Owen, B. D., 317.

CALCIUM, utilization from lactate, gluconate, sulfate and carbonate salts by young college women, 443.
Caloric and fat restriction, effect of dietary, on protein utilization, 243.
Carbohydrate and fat, influence of dietary, on reproduction and lactation in rats, 91.
Carbonate, lactate, gluconate and sulfate salts, utilization of calcium from, by young college women, 443.
Carotene, fate of lycopene in rat and its effects on utilization of, 369.
CARY, C. A. See Dryden, L. P., 509.
Casein rations, purified, relation between vitamin B_{12} deficiency and lactation in rats fed, 509.
CERECEDO, L. R., F. P. PANZARELLA, A. B. VASTA AND E. C. DERENZO. Studies on essential fatty acid deficiency in three strains of mice, 41.
CHAMBERS, W. H. Biography of Max Rubner, 3.
CHENG, A. L. S., G. D. KEYDER, L. M. BERGQUIST AND H. J. DEUEL, JR. The effect of fat level of the diet on general nutrition. IX. The relationship of radiation injury in the rat to the fat content of the diet, 161.
Chicks, effect of diet on comparative activities of pyridoxal, pyridoxamine and pyridoxine for, 531.
— utilization of homocystine, choline and related compounds, 209.
Chinchilla, nutritional studies of, with special reference to ascorbic acid and thiamine, 31.
Chloride, sodium (see Sodium chloride).

Choline, effect of vitamin B_{12} upon utilization by young poult, 201.
— homocystine and related compounds, utilization by chicks, 209.
Cobalt-deficient sheep, response to intravenously administered B_{12}, 421.
—— synthesis of certain B vitamins in, with special reference to vitamin B_{12}, 431.
College women (see Women).
COLLINS, R. A. See Schreiber, M., 125.
Congenital abnormalities, production of multiple, in young by maternal pteroylglutamic acid deficiency during gestation, 61.
Corn, effect of untreated, upon growth of rats on a niacin-tryptophan deficient diet, 453.
— relationship between protein content of, and nutritional value of the protein, 461.
Cottonseed proteins. Nutritional evaluation of food proteins by measuring availability of amino acids to microorganisms. I., 231.
CRAVENS, W. W. See Waibel, P. E., 531.
CRAVIOTO, O. Y. See Cravioto, R. O., 453.
CRAVIOTO, R. O., G. H. MASSIEU, O. Y. CRAVIOTO AND F. DE M. FIGUEROA. Effect of untreated corn and Mexican tortilla upon the growth of rats on a niacin-tryptophan deficient diet, 453.
Creatinine, diminished urinary, in vitamin E-deficient rats, 193.
— excretion, daily urinary thiamine excretion and its relation to. Thiamine metabolism of women on controlled diets. I., 297.
CUNNINGHAM, J. D. See Anderson, G. W., 539.
CURTIN, L. V., J. K. LOOSLI, J. ABRAHAM, H. H. WILLIAMS AND L. A. MAYNARD. The methionine requirement for the growth of swine, 499.

DARBY, W. J. See Meneely, G. R., 489.
DAY, H. G. See High, E. G., 369.
DERENZO, E. C. See Cerecedo, L. R., 41.
Dermatosis in weanling rats fed lactose diets. I. The influence of relative humidity and diet, 125.
DEUEL, H. J., JR. See Cheng, A. L. S., 161.
Diet(s) and relative humidity, influence of. Dermatosis in weanling rats fed lactose diets. I., 125.
— controlled, thiamine metabolism of women on. I., 297; II., 307.
— effect of level of fat in, on growth performance of dogs, 81.
—— on comparative activities of pyridoxal, pyridoxamine and pyridoxine for chicks, 531.
—— on general nutrition of fat level of. IX., 161.
— lactose, dermatosis in weanling rats fed. I., 125.
— low in L- and DL-lysine, or devoid of arginine, nitrogen balance of adult rats fed, 391.
—— protein, influence of vitamin B_{12} and antibiotics on protein and energy utilization in, 477.
— niacin-tryptophan deficient, effect of untreated corn and Mexican tortilla upon growth of rats on a, 453.
— purified, containing various levels of sodium chloride, growth of albino rat on, 489.
— relation of rate of growth to. Study of stock colony ration for albino rats, 553.
— relationship of fat content of, to radiation injury in rat, 161.
Dietary fat and caloric restriction, effect on protein utilization, 243.
—— and carbohydrate, influence on reproduction and lactation in rats, 91.
Digestion of triglycerides, intermediates formed during, 335.
DING, K. See Louhi, H. A., 297.
Dogs, effect of level of fat in diet on growth performance of, 81.
DEYDEN, L. P., A. M. HARTMAN AND C. A. CARY. The relation between vitamin B_{12} deficiency and lactation in rats fed purified casein rations, 509.
DUBÉ, R. B., E. C. JOHNSON, H. H. YÜ AND C. A. STORVICK. Thiamine metabolism of women on controlled diets. II. Daily blood thiamine values, 307.
DUBOIS, E. F., F. G. EBAUGH, JR., AND JAMES D. HARDY. Basal heat production and elimination of 13 normal women at temperatures from 22°C. to 35 C., 257.

EASTERLING, L. See Sure, B., 401.
EBAUGH, F. G., JR. See Dubois, E. F., 257.
EDWARDS, J., JR. See Rodahl, K., 359.
Elimination and production, basal heat, of 13 normal women at temperatures from 22°C. to 35°C., 257.
ELVEHJEM, C. A. See Schreiber, M., 125.
ELY, M. T. See Nasset, E. S., 391.
Energy utilization in low protein diet, influence of vitamin B_{12} and antibiotics on, 477.
ENGEL, E. See Sobel, A. E., 183.
Eskimo, basal metabolism of, 359.
EVANS, H. M. See Nelson, M. M., 61.
Excretion, creatinine, daily urinary thiamine excretion and its relation to. Thiamine metabolism of women on controlled diets. I., 297.
— urinary (see Urinary excretion).

FAT and caloric restriction, effect of dietary, on protein utilization, 243.
— and carbohydrate, influence of dietary, on reproduction and lactation in rats, 91.
— content of diet, relationship of radiation injury in rat to, 161.
— in diet, effect of level on growth performance of dogs, 81.
— level of diet, effect on general nutrition. IX. The relationship of radiation injury in the rat to the fat content of the diet, 161.
— soluble vitamins, intravenous injection of, and transfer to milk of injected vitamin A, 183.
Fatty acid deficiency, essential, in three strains of mice, 41.
Female rats (see Rats).
FIGUEROA, F. DE M. See Cravioto, R. O., 453.
Fish meals, evaluation of biological values of proteins in, by nitrogen retention method, 401.
Flora, intestinal (see Intestinal flora).
Food proteins, nutritional evaluation of, by measuring availability of amino acids to microorganisms. I., 231.
FRENCH, C. E., R. H. INGRAM, L. K. KNOBEL AND R. W. SWIFT. The influence of dietary fat and carbohydrate on reproduction and lactation in rats, 91.

GERSDORFF, C. E. F. See Horn, M. J., 231.
Gestation, production of multiple congenital abnormalities in young by maternal pteroylglutamic acid deficiency during, 61.

Gluconate, lactate, sulfate and carbonate salts, utilization of calcium from, by young college women, 443.
GRAVES, B. J. See Louhi, H. A., 297.
Growth of albino rat on a purified diet containing various levels of sodium chloride, 489.
— of rats on a niacin-tryptophan deficient diet, effect of untreated corn and Mexican tortilla upon, 453.
— of swine, methionine requirement for, 499.
— of turkey poults, effect of terramycin and certain phenylarsonic acid derivatives on, 539.
— performance of dogs, effect of level of fat in diet on, 81.
— relation of rate of, to diet. Study of stock colony ration for albino rats, 553.
GRUMMER, R. H. See Sheffy, B. E., 103.
GUGGENHEIM, K., AND R. E. OLSON. Studies of lipogenesis in certain V-vitamin deficiencies, 345.

HAMILTON, T. S. See Mitchell, H. H., 461.
HARDIN, J. O. See Hove, E. L., 193.
HARDY, J. D. See Dubois, E. F., 257.
HARSHFIELD, R. D. See Klug, H. L., 409.
HARTMAN, A. M. See Dryden, L. P., 509.
HAWTHORNE, B. E. See Louhi, H. A., 297.
Heat, effect on lysine and methionine in sunflower seed oil meal, 149.
— production and elimination, basal, of 13 normal women at temperatures from 22°C. to 35°C., 257.
HIGH, E. G., AND H. G. DAY. Fate of lycopene in the rat and its effects on the utilization of carotene and vitamin A, 369.
HILL, D. C. See Alexander, J. C., 149.
HOEKSTRA, W. G., A. L. POPE AND P. H. PHILLIPS. Response of cobalt-deficient sheep to intravenously administered B_{12}, 431. Synthesis of certain B vitamins in the cobalt-deficient sheep, with special reference to vitamin B_{12}, 421.
Homocystine, choline and related compounds, utilization by chicks, 209.
HORN, M. J., A. E. BLUM, M. WOMACK AND C. E. F. GERSDORFF. Nutritional evaluation of food proteins by measuring availability of amino acids to microorganisms. I. Cottonseed proteins, 231.
HOVE, E. L., AND J. O. HARDIN. Diminished urinary creatinine in vitamin E-deficient rats, 193.
HSU, C. See Steele, B. F., 49.
HUBBELL, R. B., AND W. A. KREHL. The relation of the rate of growth to the diet. A study of a stock colony ration for albino rats, 553.
Human (see Man).
Humidity, relative, and diet, influence of. Dermatosis in weanling rats fed lactose diets. I., 125.

INGRAM, R. H. See French, C. E., 91.
Injury, radiation, in rat, relationship to fat content of diet, 161.
Intermediates formed during digestion of triglycerides, 335.
Intestinal flora of turkey poults, effect of terramycin and certain phenylarsonic acid derivatives on, 539.
Intraperitoneal rat test, evaluation of nitrogen source materials by, 377.
Intravenous injection of fat-soluble vitamins and transfer to milk of injected vitamin A, 183.
Intravenously administered B_{12}, response of cobalt-deficient sheep to, 421.

JOHNSON, E. C. See Dubé, R. B., 307.
JUKES, T. H., AND E. L. R. STOKSTAD. Further observations on the utilization of homocystine, choline and related compounds by chicks, 209.

KING, K. W., AND F. S. ORCUTT. Nutritional studies of the chinchilla, with special reference to ascorbic acid and thiamine, 31.
KLUG, H. L., R. D. HARSHFIELD, R. M. PENGRA AND A. L. MOXON. Methionine and selenium toxicity, 409.
KNOEBEL, L. K., AND A. BLACK. The influence of vitamin B_{12} and antibiotics on protein and energy utilization in a low protein diet, 477.
— See French, C. E., 91.
KOSKO, S. H. See Dubé, R. B., 307.
KRATZER, F. H. The effect of vitamin B_{12} upon the utilization of choline and betaine by the young poult, 201.
KREHL, W. A. See Hubbell, R. B., 553.
KRYDER, G. D. See Cheng, A. L. S., 161.

LACTATE, gluconate, sulfate and carbonate salts, utilization of calcium from, by young college women, 443.
Lactation and vitamin B_{12} deficiency, relation between, in rats fed purified casein rations, 509.
— in rats, influence of dietary fat and carbohydrate on, 91.
— nitrogen constituents of sow's milk as affected by stage of, 103.
Lactose diets, dermatosis in weanling rats fed. I., 125.
Lipogenesis in certain B-vitamin deficiencies, 345.
Liver storage of vitamin A by male and female rats, 13.
LOOSLI, J. K. See Curtin, L. V., 499.
LOUHI, H. A., H. H. YÜ, B. E. HAWTHORNE AND C. A. STORVICK. Thiamine metabolism of women on controlled diets. I. Daily urinary thiamine excretion and its relation to creatinine excretion, 297.
Lycopene, fate in rat and effects on utilization of carotene and vitamin A, 369.
Lysine (D- and DL-), nitrogen balance of adult rats fed diets low in, 391.
— in sunflower seed oil meal, effect of heat on, 149.

MALE rats (see Rats).
Man, ascorbic acid nutriture in. I., 49.
MARTIN, J. B. See Mattson, F. H., 335.
MASSIEU, G. H. See Cravioto, R. O., 453.
Maternal pteroylglutamic acid deficiency during gestation, production of multiple congenital abnormalities in young by, 61.
MATTSON, F. H., J. H. BENEDICT, J. B. MARTIN AND L. W. BECK. Intermediates formed during the digestion of triglycerides. 335.
MAX RUBNER (1854–1932), 3.
MAYNARD, L. A. See Curtin, L. V., 499.
MCFARLAND, S. S. See Dubé, R. B., 307.
MCHENRY, E. W. See Beaton, J. R., 325.
Mead Johnson vitamin B-complex award, presentation, 1952, 121.

MENEELY, G. R., R. G. TUCKER AND W. J. DARBY. Chronic sodium chloride toxicity in the albino rat. I. Growth on a purified diet containing various levels of sodium chloride, 489.
Metabolism, basal, of the Eskimo, 359.
— thiamine, of women on controlled diets. I. Daily urinary thiamine excretion and its relation to creatinine excretion, 297; II. Daily blood thiamine values, 307.
— (tyrosine) and blood levels of ascorbic acid during ascorbic acid depletion and repletion, 49.
Methionine and selenium toxicity, 499.
— in sunflower seed oil meal, effect of heat on, 149.
— requirement for growth of swine, 499.
Mexican tortilla, effect upon growth of rats on a niacin-tryptophan deficient diet, 539.
Mice, essential fatty acid deficiency in three strains of, 41.
Microorganisms, availability of amino acids to, nutritional evaluation of food proteins by measuring. I., 231.
Milk of swine fed supplemental levels of pantothenic acid, pantothenic acid content of, 317.
— sow's, nitrogen constituents of, as affected by ration and stage of lactation, 103.
— transfer to, of injected vitamin A, intravenous injection of fat-soluble vitamins and, 183.
MITCHELL, H. H., T. S. HAMILTON AND J. R. BEADLES. The relationship between the protein content of corn and the nutritional value of the protein, 461.
MOXON, A. L. See Klug, H. L., 409.

NASSET, E. S., AND M. T. ELY. Nitrogen balance of adult rats fed diets low in L- and DL-lysine, or devoid of arginine, 391.
NELSON, M. M., C. W. ASLING AND H. M. EVANS. Production of multiple congenital abnormalities in young by maternal pteroylglutamic acid deficiency during gestation, 61.
Niacin-tryptophan deficient diet, effect of untreated corn and Mexican tortilla upon growth of rats on a, 453.
NIÑO-HERRERA, H. See Schreiber, M., 125.
Nitrogen balance of adult rats fed diets low in L- and DL-lysine, or devoid of arginine, 391.
— constituents of sow's milk as affected by ration and stage of lactation, 103.
— retention method, evaluation of biological values of proteins in fish meals by, 401.
— source materials, evaluation by intraperitoneal rat test, 377.
Normal women (see Women, normal).
Nutrition, Borden award in (see Borden award in nutrition).
— general, effect of fat level of diet on. IX., 161.
Nutritional evaluation of food proteins by measuring availability of amino acids to microorganisms. I. Cottonseed proteins, 231.
— studies of the chinchilla, with special reference to ascorbic acid and thiamine, 31.
— value of the protein, relationship between protein content of corn and, 461.
Nutriture, ascorbic acid, in the human. I., 49.

OIL meal (sunflower seed), effect of heat on lysine and methionine in, 149.
OLSON, R. E. See Guggenheim, K., 345.
ORCUTT, F. S. See King, K. W., 31.
Osborne and Mendel award, announcement, 1953, 124, 295; presentation, 1952, 121.
OWEN, B. D., AND J. P. BOWLAND. The pantothenic acid content of the blood and milk of swine fed supplemental levels of the vitamin, 317.

PANTOTHENIC acid content of blood and milk of swine fed supplemental levels of the vitamin, 317.
PANZARELLA, F. P. See Cerecedo, L. R., 41.
PATTON, M. B., AND T. S. SUTTON. The utilization of calcium from lactate, gluconate, sulfate and carbonate salts by young college women, 443.
PENGRA, R. M. See Klug, H. L., 409.
Peptone and amino acid ingestion, effect upon concentrations of tissue amino acids, 139.
Phenylarsonic acid derivatives, effect of certain, on growth and intestinal flora of turkey poults, 539.
PHILLIPS, P. H. See Hoekstra, W. G., 431, 421; Sheffy, B. E., 103.
PIERCE, Z. H. See Steele, B. F., 49.
POPE, A. L. See Hoekstra, W. G., 431, 421.
Poults, turkey (see Turkey poults).
Proceedings of the American Institute of Nutrition (see American Institute of Nutrition).
Protein(s) content of corn and nutritional value of the protein, relationship between, 461.
— cottonseed. Nutritional evaluation of food proteins by measuring availability of amino acids to microorganisms. I., 231.
— food, nutritional evaluation of, by measuring availability of amino acids to microorganisms. I., 231.
— in fish meals, evaluation of biological values of, by nitrogen retention method, 401.
— relationship between protein content of corn and nutritional value of the, 461.
— utilization, effect of dietary fat and caloric restriction on, 243.
—— in low protein diet, influence of vitamin B_{12} and antibiotics on, 477.
Pteroylglutamic acid deficiency, maternal, during gestation, production of multiple congenital abnormalities in young by, 61.
Purified casein rations, relation between vitamin B_{12} deficiency and lactation in rats fed, 509.
— diet containing various levels of sodium chloride, growth of albino rat on, 489.
Pyridoxal, pyridoxamine and pyridoxine, effect of diet on comparative activities of, for chicks, 531.
Pyridoxamine, pyridoxal and pyridoxine, effect of diet on comparative activities of, for chicks, 531.
Pyridoxine deficient rats, factors affecting development of acrodynia in, 325.
— pyridoxal and pyridoxamine, effect of diet on comparative activities of, for chicks, 531.

RADIATION injury in rat, relationship to fat content of diet, 161.
Rat(s), adult, fed diets low in L- and DL-lysine, or devoid of arginine, nitrogen balance of, 391.
— albino, chronic sodium chloride toxicity in. I., 489.
—— study of stock colony ration for. Relation of rate of growth to diet, 553.
— fate of lycopene in, and its effects on utilization of carotene and vitamin A, 369.

— fed purified casein rations, relation between vitamin B_{12} deficiency and lactation in, 509.
— influence of dietary fat and carbohydrate on reproduction and lactation in, 91.
— male and female, liver storage of vitamin A by, 13.
— on a niacin-tryptophan deficient diet, effect of untreated corn and Mexican tortilla upon growth of, 453.
— pyridoxine-deficient, factors affecting development of acrodynia in, 325.
— relationship of radiation injury in, to fat content of diet, 161.
— test, intraperitoneal, evaluation of nitrogen source materials by, 377.
— vitamin E-deficient, diminished urinary creatinine in, 193.
— weanling, fed lactose diets, dermatosis in. I., 125.
— young, production of multiple congenital abnormalities in, by maternal pteroylglutamic acid deficiency during gestation, 61.
Ration(s), nitrogen constituents of sow's milk as affected by, 103.
— purified casein, relation between vitamin B_{12} deficiency and lactation in rats fed, 509.
— (stock colony), study of, for albino rats. Relation of rate of growth to diet, 509.
Reproduction in rats, influence of dietary fat and carbohydrate on, 91.
RODAHL, K. WITH TECHNICAL ASSISTANCE OF JAMES EDWARDS, JR. Basal metabolism of the Eskimo, 359.
ROSENBERG, A. See Sobel, A. E., 183.
ROSENTHAL, H. L. The effect of dietary fat and caloric restriction on protein utilization, 243.
RUBNER, MAX (1854–1932), 3.

SALTS (lactate, gluconate, sulfate and carbonate), utilization of calcium from, by young college women, 443.
SCHAD, J. S. See Arnold, A., 377.
SCHREIBER, M., R. A. COLLINS, H. NIÑO-HERRERA AND C. A. ELVEHJEM. Dermatosis in weanling rats fed lactose diets. I. The influence of relative humidity and diet, 125.
SCHWEIGERT, B. S. See Siedler, A. J., 81.
Seed, sunflower (oil meal), effect of heat on lysine and methionine in, 149.
Selenium and methionine toxicity, 409.
SHAHANI, K. M. See Sheffy, B. E., 103.
Sheep, cobalt-deficient, response to intravenously administered B_{12}, 421.
—— synthesis of certain B vitamins in, with special reference to vitamin B_{12}, 421.
SHEFFNER, A. L., AND O. BERGEIM. The effect of peptone and amino acid ingestion upon the concentrations of tissue amino acids, 139.
SHEFFY, B. E., K.M. SHAHANI, R. H. GRUMMER, P. H. PHILLIPS AND H. H. SOMMER. Nitrogen constituents of sow's milk as affected by ration and stage of lactation, 103.
SIEDLER, A. J., AND B. S. SCHWEIGERT. Effect of the level of fat in the diet on the growth performance of dogs, 81.
SLINGER, S. J. See Anderson, G. W., 539.
SNELL, E. E. See Waibel, P. E., 531.
SOBEL, A. E., A. ROSENBERG AND E. ENGEL. Intravenous injection of fat-soluble vitamins and transfer to milk of injected vitamin A, 183.
SODERSTROM, G. F. See Dubois, E. F., 257.
Sodium chloride, growth of albino rat on a purified diet containing various levels of, 489.
—— toxicity, chronic, in albino rat. I. Growth on a purified diet containing various levels of sodium chloride, 489.
SOMMER, H. H. See Sheffy, B. E., 103.
Sow's milk, nitrogen constituents of, as affected by ration and stage of lactation, 103.
STEELE, B. F., C. H. HSU, Z. H. PIERCE AND H. H. WILLIAMS. Ascorbic acid nutriture in the human. I. Tyrosine metabolism and blood levels of ascorbic acid during ascorbic acid depletion and repletion, 49.
STEVENS, E. I. See Dubois, E. F., 257.
Stock colony ration for albino rats, study of. Relation of rate of growth to diet, 489.
STOKSTAD, E. L. R. See Jukes, T. H., 209.
Storage, liver, of vitamin A by male and female rats, 13.
STORVICK, C. A. See Dubé, R. B., 307; Louhi, H. A., 297.
Sulfate, lactate, gluconate and carbonate salts, utilization of calcium from, by young college women, 443.
Sunflower seed oil meal, effect of heat on lysine and methionine in, 149.
SURE, B., AND L. EASTERLING. Evaluation of the biological values of the proteins in fish meals by the nitrogen retention method, 401.
SUTTON, T. S. See Patton, M. B., 443.
SWIFT, R. W. See French, C. E., 91.
Swine fed supplemental levels of pantothenic acid, pantothenic acid content of blood and milk of, 317.
— methionine requirement for growth of, 499.

TEMPERATURES from 22°C. to 35°C., basal heat production and elimination of 13 normal women at, 257.
Terramycin, effect on growth and intestinal flora of turkey poults, 539.
Test, intraperitoneal rats, evaluation of nitrogen source materials by, 377.
Thiamine excretion, daily urinary, and its relation to creatinine excretion. Thiamine metabolism of women on controlled diets. I., 297.
— metabolism (see Metabolism, thiamine).
— nutritional studies of the chinchilla, with special reference to, 31.
— values, daily blood. Thiamine metabolism of women on controlled diets. II., 307.
Tissue amino acids, effect of peptone and amino acid ingestion upon concentrations of, 139.
Tortilla, Mexican, effect upon growth of rats on a niacin-tryptophan deficient diet, 539.
Toxicity, chronic sodium chloride, in albino rat. I., 489.
— methionine and selenium, 409.
Triglycerides, intermediates formed during digestion of, 335.
Tryptophan-niacin deficient diet, effect of untreated corn and Mexican tortilla upon growth of rats on a, 539.
TUCKER, R. G. See Meneely, G. R., 489.
Turkey poult(s), effect of terramycin and certain phenylarsonic acid derivatives on growth and intestinal flora of, 539.
—— young, effect of vitamin B_{12} upon utilization of choline and betaine by, 201.
Tyrosine metabolism (see Metabolism, tyrosine).

URINARY creatinine, diminished, in vitamin E-deficient rats, 193.
— thiamine excretion, daily, and its relation to creatinine excretion. Thiamine metabolism of women on controlled diets. I., 297.

VASTA, A. B. See Cerecedo, L. R., 41.

Vitamin(s) A, fate of lycopene in rat and its effects on utilization of, 369.
— — liver storage by male and female rats, 13.
— — transfer to milk of injected, intravenous injection of fat-soluble vitamins and, 183.
— B, synthesis of certain, in cobalt-deficient sheep, with special reference to vitamin B_{12}, 431.
— B_{12} deficiency and lactation, relation between, in rats fed purified casein rations, 509.
— — effect upon utilization of choline and betaine by young poult, 201.
— — influence on protein and energy utilization in low protein diet, 461.
— — response of cobalt-deficient sheep to intravenously administered, 421.
— — synthesis of certain B vitamins in cobalt-deficient sheep, with special reference to, 421.
— E-deficient rats, diminished urinary creatinine in, 193.
— intravenous injection of fat-soluble, and transfer to milk of injected vitamin A, 183.

WAIBEL, P. E., W. W. CRAVENS AND E. E. SNELL. The effect of diet on the comparative activities of pyridoxal, pyridoxamine and pyridoxine for chicks, 531.
Weanling rats (see Rats, weanling).
WILLIAMS, H. H. See Curtin, L. V., 499; Steele, B. F., 49.
WOMACK, M. See Horn, M. J., 231.
Women on controlled diets, thiamine metabolism of. I., 297; II., 307.
— 13 normal, basal heat production and elimination of, at temperatures from 22°C. to 35°C., 257.
— young college, utilization of calcium from lactate, gluconate, sulfate and carbonate salts by, 443.

YOUNG poult (see Turkey poult, young).
— rats (see Rats, young).
— women (see Women, young).
YÜ, H. H. See Dubé, R. B., 307; Louhi, H. A., 297.